UNIVERSITY OF MINHO
DEPARTAMENT OF BIOLOGICAL ENGINEERING

Candida dubliniensis versus *Candida albicans* adhesion and biofilm formation

Dissertation for PhD degree in Chemical and Biological Engineering

SUPERVISOR: Prof. Rosário Oliveira

CO-SUPERVISOR: Dr. Joana Azeredo

MARIANA CONTENTE RANGEL HENRIQUES

AKNOWLEDGMENTS

"O mestre que caminha à sombra do templo entre os discípulos, não reparte a sua sabedoria mas antes a sua fé e o seu amor" (Drª Rosário Oliveira)

"e todo o trabalho é vazio menos onde há amor" (Drª Joana Azeredo)

"e se não puderdes trabalhar com amor, mas apenas aborrecidos, mais vale abandonares o trabalho"(Drª Maria João, Pilar, Olívia, Sanna, Salomé, Lúcia, Fernando, Nuno A., Manuel, Nuno C., André, Kelly, Lívia, Bárbara, Belinda, Ana Margarida, Ana Fagos, Cláudia)

"quando trabalhais, sois uma flauta através da qual o murmúrio das horas se transforma em música"(Bárbara)

"trabalhar com amor é tecer o pano com fios tirados do vosso coração" (Belinda)

"o vosso amigo…
… é a resposta às vossas necessidades"(Rogério, Cristina, Raquel, Pedro, Xana, Ana, Alcino, Paulo, Nelson, Hugo e mitras)

…é o vosso campo, semeado com amor e ceifado com reconhecimento"(Adriano e Hugo)

… buscai-o sempre para as horas vivas" (Ana Fagos)

"e na doçura da vossa amizade haja também riso e os prazeres partilhados" (Sanna e Salomé)

"porque no orvalho das pequenas coisas o coração encontra a sua manhã e a sua frescura"(Cristina)

"quando vos separardes do vosso amigo não vos aflijais, porque aquilo que mais nele amais pode ficar claro na sua ausência" (Ana Margarida)

"porque na amizade, todos os pensamentos, todos os desejos, todas as expectativas, nascem sem palavras e partilham-se numa alegria calada" (Olívia)

"porque vindes a ele com a vossa fome e procurais nele a paz"(Eduardo)

"o hoje é só memória do dia de ontem e o amanhã é sonho de hoje"(Mariana M., Kuki, David, João Luís, Patrícia, Mariana B., Margarida, Bruno, Pedro e Mocamfe)

" e um barco sem leme pode navegar sem rumo entre perigosos recifes sem, contudo ir a pique" (Ângela Maia)

"o vento não fala mais docemente ao carvalho gigante do que à humilde folhinha de erva"(D. Ju)

"Acordar de manhã com um coração alado e agradecer outro dia de amor" (Helena e Carlos)

"descansar ao meio dia e meditar no êxtase do amor" (Luís e Susana)

"voltar a casa ao crepúsculo com gratidão"(Guida)

"adormecer tendo no coração uma prece pelo bem amado"(Manuel, Nesa, Rui, Guida, Miguel, Pia, Quim e Paula)

"e um canto de louvor na boca"(Miguel, Luísa, Teresa, Gonçalo, Pedro, Catarina e Mi)

"quando o amor vos fizer sinal segui-o" (Benedita e Ricardo)

"quando as suas asas vos envolverem entregai-vos" (Pedro e Isabel)

In "O Profeta" de Khalil Gibran

Não poderia contudo deixar de agradecer aqueles que tornaram viável este trabalho por toda a disponibilidade demonstrada:

À Drª Célia Pais e Drª Judite Almeida , do departamento de Biologia da Universidade do Minho, por terem tão gentilmente cedido os dois isolados clínicos de *Candida albicans*.

À Drª Ascensão Lopes que me ensinou e permitiu fazer as pastilhas de hidroxilapatite.

Ao professor Luís de Melo pela disponibilidade de utilização do aparelho de medição de ângulos de contacto no departamento de Engenharia Química da Universidade do Porto. E em especial à Roxane por todo o apoio e momentos de descontracção.

Ao Dr. Manuel Vilanova do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto pela cedência de uma estirpe de referência de *Candida albicans*.

To Dr. Quirynen and Wim for the possibility to learn the techniques of cell culture and work in their lab at Leuven University. Also to Martine and Caroline for all the friendship.

À Drª Elsa Anes, da Faculdade de Farmácia da Universidade de Lisboa por tão gentilmente me ter permitido executar os ensaios de adesão a células epiteliais no seu laboratório.

Ao Sr. Santos pelas centenas de cupões de acrílico cortados.

Não posso deixar de referir o Departamento de Engenharia Biológica (que tem sido uma segunda casa nos últimos anos) e todos os que dele fazem parte.

RESUMO

A espécie *Candida* é constituída por leveduras comensais do Homem. A espécie mais conhecida e mais virulenta é a *Candida albicans*. Em 1995 foi identificada uma nova espécie, *Candida dubliniensis*, que durante muito tempo foi confundida com *Candida albicans.* Estas duas espécies possuem propriedades fenotípicas e genotípicas semelhantes. No entanto, como a *Candida dubliniensis* não foi tão estudada como a *Candida albicans* é importante conhecer alguns dos seus aspectos característicos. A cavidade oral foi o habitat escolhido no presente estudo, dado que a *Candida dubliniensis* foi descoberta na boca de indivíduos imuno-suprimidos.

O principal objectivo deste trabalho foi a comparação das duas espécies, em termos de propriedades superficiais, da capacidade de adesão a materiais inertes e células epiteliais e de formação de biofilmes e da susceptibilidade a concentrações sub-inibitórias de agentes antifúngicos.

Em cada ensaio foram usadas duas estirpes de cada uma das espécies em estudo e saliva artificial para simular as condições da cavidade oral.

As células das leveduras e as superfícies inertes foram caracterizadas relativamente às propriedades físico-químicas relevantes no processo de adesão (tensão superficial, grau de hidrofobicidade e potencial zeta), tendo sido também determinadas as respectivas composições elementares por espectroscopia de raios X (XPS).

Nos ensaios de adesão foram utilizadas como superfícies: acrílico auto-polimerizável, usado em próteses dentárias e hidroxilapatite (HAP) que simula o esmalte dos dentes. Estes ensaios decorreram durante uma hora e foram efectuados em água e em saliva artificial. O número de células aderentes foi determinado por enumeração directa, após coloração fluorescente das amostras.

Efectuaram-se também ensaios de adesão das leveduras a células epiteliais, tendo-se recorrido a uma linha celular (HeLa) e a enumeração directa das células aderentes após coloração de Gram.

Os biofilmes foram formados em meio Sabouraud (SDB) e em meio de saliva artificial (saliva artificial suplementada com nutrientes). A evolução da formação dos biofilmes foi avaliada em termos de biomassa total, usando-se para tal coloração com violeta

cristal, e em termos de actividade, recorrendo à reacção característica com XTT (2,3-bis(2-metoxi-4-nitro-5-sulfofenil)-5-na -2H-hidróxido de tetrazólio).
O estudo da susceptibilidade a antifungicos foi feito recorrendo a um agente fungicida, a anfotericina B e a um agente fungistático, o fluconazol. As células cresceram em meio de saliva artificial suplementado com concentrações sub-inibitórias destes agentes e foram utilizadas em ensaios de adesão (1 h em água), de formação de biofilme (24 h em SDB ou saliva artificial) e de determinação do perfil de formação de biofilme (até 78 h em presença de concentrações sub-inibitórias dos agents antifúngicos). Tal como no caso anterior foram determinadas a biomassa total e a actividade dos biofilmes.

Dos resultados obtidos relativamente às propriedades superficiais foi possível concluir que não existiam diferenças significativas entre as diversas estirpes, independentemente do meio (água, saliva artificial, solução salina ou meios de saliva artificial suplementados com fluconazol e anfotericina B).
Uma das principais conclusões obtidas neste trabalho é que a adesão das leveduras a superfícies inertes é independente da estirpe e da espécie, verificando-se o contrário na adesão a células epiteliais e na formação de biofilme.
Não foi detectado nenhum efeito significativo, tanto na adesão das leveduras ao acrílico como na formação de biofilme, quando as células cresceram em concentrações sub-inibitórias dos agentes antifúngicos.

ABSTRACT

Candida species are opportunistic yeasts that colonize the human body. Among the more well known and more virulent is Candida albicans. Although for several years, until 1995, another species, Candida dubliniensis, was mismatched with that one. Both Candida species possess similar phenotypic and genotypic characteristics. As this species has not been fully studied, it became important to understand its behaviour and its properties. Candida dubliniensis is mainly found in the mouth of some HIV patients. So, its behaviour in the oral cavity was chosen as the objective of the present work.

The main goal of this work was the comparison of those two species in terms of the ability to adhere to inert surfaces and epithelial cells, to form biofilms and susceptibility to antifungal agents.

The characteristics and behaviours under concern were assayed in two strains of each species. Artificial saliva solution was used in order to simulate the oral conditions.
Yeast cells and inert surfaces were characterized in terms of physico-chemical surface properties (surface tension parameters, degree of hydrophobicity and zeta potential) as well as elemental surface composition determined by X-ray Photoelectron Spectroscopy.
Adhesion to inert surfaces was assayed on acrylic, that is commonly used in prosthetic devices and hydroxyapatite (HAP), which mimics tooth enamel. The adhesion was performed during one hour, both in ultrapure water and artificial saliva. The number of adhered cells was determined by direct enumeration using fluorescence microscopy.
Adhesion of yeast cells was also performed to epithelial cells (HeLa cell line) and was quantified by direct enumeration, after Gram staining.
Concerning the biofilm formation, the profiles of biofilm evolution were determined for biofilms formed on acrylic and grown in Sabouraud dextrose broth or artificial saliva growth medium. The biofilm formation was evaluated by quantifying total biomass (crystal violet staining) and activity (XTT/formazan salts formation).
Two different antifungal agents were used, one fungistatic – fluconazole – and one fungicidal – amphotericin B. The yeast cells response to subinhibitory concentrations of both antifungal agents was analysed in terms of adhesion and biofilm formation

(either in SDB or artificial saliva growth medium), using cells grown in media containing those agents. The influence of subinhibitory concentrations of the antifungal agents was also studied in biofilm formation along 78 h in the presence of the antifungal agents. The assessment was performed in terms of biofilm biomass and activity.

Considering cell surface properties (surface tension parameters, degree of hydrophobicity), there were no significative differences among the four strains assayed, neither when these properties were measured with cells conditioned by different solutions (water, artificial saliva solution, saline solution or after growth in subMIC concentrations of fluconazole and amphotericin B).

The results obtained lead to the conclusion that although adhesion to inert surfaces is not strain dependent, adhesion to epithelium and biofilm formation are species and strain dependent.

The effect of sub inhibitory concentrations of the antifungal agents on the growth of yeast cells and consequent extent of adhesion and biofilm formation was not notorious.

NOMENCLATURE

Symbols

p – significance value

r^2 – correlation factor

Ra – roughness (μm)

t – time (min)

T – temperature (°C)

θ_w – water contact angle (°)

θ_f – formamide contact angle (°)

θ_b – α-bromonaphtalene contact angle (°)

γ_i^{LW} - apolar (Lifshitz-van der Waals) surface tension parameter of a substance *i* (mJ m^{-2})

γ_i^{AB} - polar (Lewis acid-base) surface tension parameter of a substance *i* (mJ m^{-2})

γ_i^{+} - electron acceptor surface tension parameter of a substance *i* (mJ m^{-2})

γ_i^{-} - electron donor surface tension parameter of a substance *i* (mJ m^{-2})

ΔG_{sws}^{tot} - total free energy variation between two entities of a given surface (s) immersed in water (w) (mJ m^{-2})

ΔG_{sws}^{LW} - apolar component of the free energy variation between two entities of a given surface (s) immersed in water (w) (mJ m^{-2})

ΔG_{sws}^{AB} - polar component of the free energy variation between two entities of a given surface (s) immersed in water (w) (mJ m^{-2})

ζ - zeta potential (mV)

Abbreviations

Abs – Absorbance

AIDS – Acquired mmunodeficiency syndrome

ANOVA – Analysis of variance

ATCC – American type culture collection

ATP – Adenosine tri-phosphate

CBS – Centraalbureau voor Schimmelcultures

CFU – Colony forming unit

CSH – Cell surface hydrophobicity

CV – Crystal violet

BEC – Buccal epithelial cells

DNA – Desoxiribonucleic acid

DMSO – Dimethyl sulfoxide

ECM – Extracellular matrix

EMEM – Minimal essential medium with Eagle´s salts

EP – Extracellular polymers

HAP – Hydroxyapatite

HMA – Hydrophobic microsphere assay

MATH – Microbial adhesion to hydrocarbons

MFC – Minimal fungal concentration

MIC – Minimal inhibitory concentration

MTT – 3-(4,5-dimethylthiazol-2-thiazyl)-2,5-diphenyltetrazolium bromide

NCCLS – National Committee for Clinical Laboratory Standards

PBS – Phosphate saline buffer

PMS – Phenazine methosulfate

RSA – Rapid susceptibility assay

SAP – Secreted aspartyl protease

SDA - Sabouraud dextrose agar

SDB – Sabouraud dextrose broth

SEM – Scanning electron microscopy

SPSS – Statistical package for the social sciences

subMIC – Subinhibitory minimal concentration

VEC – Vaginal epithelial cells

XPS – X-ray photoelectron microscopy

XTT – 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 - INTRODUCTION

Oral candiddosis is one of the most common conditions encountered in patients with HIV infection, developing in over 80% of these individuals at some time during their illness. In noncompromised patients, this infection can often be eradicated with a short course of topical or oral treatment with an azole compound. However, because of the profound and sustained immunosupression in patients with AIDS, persistent or recurrent oral infection with *Candida albicans* is common and long courses of antifungal treatment are required if remission from infection is to be achieved and sustained (Jacobs and Nall, 1997). Besides *Candida albicans*, other species have gained importance clinically, as, *C. glabrata*, *C. krusei* and *C. dubliniensis*. On account of this, the study of the mechanisms of adhesion and biofilm formation or resistance to antifungal agents of *Candida* species became very important in the last years.

According to Odds (1987), most people usually carry a single strain of *Candida* at different body sites for a long time. However, it has been shown that a few individuals may harbour more than one strain or species of *Candida* at the same time, and that in hospitalized and immunocompromised patients this occurs more commonly (McCullough *et al.*, 1996).

The fact that makes *Candida* simply to remain as a commensal or to proliferate, invading tissues and producing candidiasis is determined by the change in the environment of the host. The commensal relationship is dependent on the maintenance of host tissue integrity with normal microbial flora as well as on an intact immune system. As long as these host conditions are maintained, mucosal candidiasis is not observed clinically. However, a breakdown in anatomic integrity or a change in the resident microbial flora can lead to environmental conditions that are favourable for the growth of *Candida* spp. with potential for host invasion by the fungus. Such environmental conditions in conjunction with an imbalance of host cytokine response can lead to increased tissue colonization and fungal overload resulting in mucosal candidiasis (Calderone, 2002c).

The infections produced range from the superficial to the systemic. The latter type is mainly observed in individuals with immunological deficiencies and represents an important clinical problem (Pla *et al.*, 1996). *Candida* species can colonize different body sites as oral cavity, oesophagus, gastrointestinal tract, genital tract and skin (Figure 1.1).

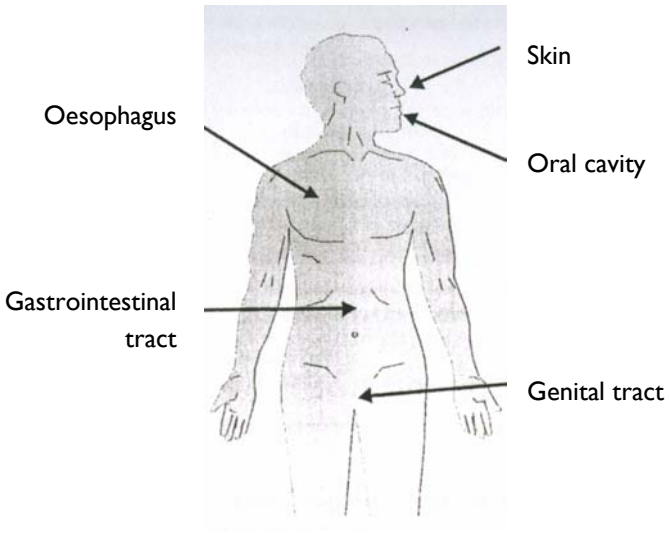


Figure 1.1 – Different body sites that can be colonized by *Candida* species.

1.1 – *Candida* species

Although *Candida albicans* is the most important pathogen, other species, as *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. kefyr* and *C. dubliniensis*, have gained clinically importance. *Candida dubliniensis* was the last *Candida* species identified and it has been mismatched for some years with *Candida albicans*. As they have some different characteristics there are several methods to differentiate them.

1.1.1 – *Candida albicans*

It took 200 years before the etiological agent of trush, the first type of candidiasis that was described, was identified correctly. Berg (1846; Calderone, 2002b), in 1846, based in experimental observations, considered that trush was caused by a fungus. He concluded that healthy children were less prone to trush, although epidemics could occur associated with communal feeding bottles and, most important, "descriptions of the disease unsupported by demonstration of the fungus could not substantiate the diagnosis". This scientific approach of Berg was one of the most important contributions to the study of trush. After his studies it became clear that the disease had several different manifestations in addition to the oral infection. Robin (1853), was the first to use the term *albicans* (which means "to whiten") when he reclassified the

fungus responsible for the trush, already classified by Gruby (1842) as a species of *Sporotrichum*, as *Oidium albicans*. Only some years latter, the name *Candida* was used by Berkhout (1923). The name was derived from the Latin phrase *toga Candida*, which was used to describe a special white robe worn by Candidates for the Roman Senate. The derived name is probably in reference to the whitish colonies on agar (Figure 1.2) or the oral lesions of aphthae or trush.

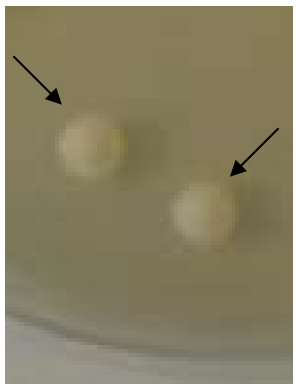


Figure 1.2 – Image of two colonies of *Candida albicans* in a plate with Sabouraud Dextrose Agar.

The binomial *Candida albicans* was officially adopted in 1954 at the Eighth Botanical Congress (Calderone, 2002b). The genus *Candida* is within the class Deuteromyces and has been described as a "taxonomic pit" into which the yeasts without a known sexual stage or other remarkable phenotypic character have been thrown (Odds, 1987). Molecular biological approaches to the study of *Candida albicans* were first published during the 1980s; the organism was proven to have a diploide genome, and natural heterozygosity was described in the isolates (Calderone, 2002b; Whelan and Magee, 1981).

One of the main characteristics of *Candida albicans* is the germ tube formation. The germ tube can be induced in complex media, chemically defined media and serum. Germination can be favoured by temperatures higher than 35 °C and a pH between 6.5 and 7 or slightly alkaline.

1.1.2 – *Candida dubliniensis*

In the early 20th century, *Candida albicans* was considered the only *Candida* species of real medical importance, although *C. stellatoidea* (now considered synonymous with *C. albicans*), *C. parapsilosis*, *C. tropicalis* and *C. guillermondii* were considered occasional pathogens (Hazen, 1995; Moran *et al.*, 2002). However, during the 1980s and 1990s, the frequency with which these organisms were recovered from sites of infection increased and species that were previously considered non-pathogenic, such as *C. glabrata*, *C. krusei* and *C. lusitanae*, emerged as pathogens (Pfaller, 1996; Pfaller and Wenzel, 1992). It was only in 1995 (Sullivan *et al.*, 1995) that *Candida dubliniensis* was identified as a non-*Candida albicans Candida* (NCAC) species. The reasons for this shift in the epidemiology of *Candida* infections may be linked to the general increase in the incidence of mycoses from the 1960s onward (Jarvis, 1995; Moran *et al.*, 2002; Wenze, 1995).

The characteristics of *Candida dubliniensis* are very similar to the ones of *Candida albicans*, which made them to be mismatched for all these years. Nevertheless, there are some differences between these two *Candida* species, which enable their differentiation. Considering the phenotypic characteristics, *C. dubliniensis* grows well at 30 and 37°C, as well as *C. albicans*, on culture media routinely used to grow *Candida* species (Sullivan *et al.*, 1995). However, unlike *C. albicans*, isolates of *C. dubliniensis* grow poorly or not at all at 42°C (Coleman *et al.*, 1997; Sullivan *et al.*, 1997; Sullivan *et al.*, 1995). Both species form creamy white colonies in Sabouraud agar (the most used medium to grow *Candida* species) but in CHROMagar Candida these strains present colonies with different colours. One significant difference between *C. albicans* and *C. dubliniensis* is the inability of the latter to express β-glucosidase activity (Schoofs *et al.*, 1997; Sullivan *et al.*, 1997). In comparison with isolates of *C. albicans*, isolates of *C. dubliniensis* have been demonstrated to produce increased levels of extracellular proteinase and to have an increase ability to adhere to buccal epithelial cells (McCullough *et al.*, 1995). Two common characteristics of both species are the germ tube and chlamydospores formation, features usually considered in the diagnostic of *C. albicans* (Schoofs, 1997; Sullivan *et al.*, 1997; Sullivan *et al.*, 1995; Sullivan and Coleman, 1998).

Regarding the genotypic characteristics, one of the earliest clues which suggested that *C. dubliniensis* isolates represented a group of organisms distinct from typical *C. albicans* arose from DNA fingerprint studies. *C. dubliniensis* isolates can also be differentiated from other *Candida* species by a variety of other molecular biology-based techniques (Sullivan and Coleman, 1998).

The recent emergence of *Candida dubliniensis*, as an opportunistic pathogen in the oral cavities of HIV-infected individuals and AIDS patients, is in part due to its ability to develop stable fluconazole resistance, which is often used to treat these patients.

1.1.3 – Strain differentiation

The differentiation between *Candida albicans* and *Candida dubliniensis* has been largely studied in the last years and several methods have been developed. The strains differentiation can be achieved with phenotypic or genetic methods. The former can include the serotyping (Brawner *et al.*, 1992), the resistogram typing (Hunter and Fraser, 1987), the yeast "killer toxin" typing, morphotyping, biotyping and protein typing (McCullough *et al.*, 1996). Among the genetic methods are the use of species-specific DNA probes (Elie *et al.*, 1998), detection of secreted aspartic proteinase genes (Flahaut *et al.*, 1998) and fluorescent in situ hybridazition (Lischewski *et al.*, 1997).

Several tests based on the growing factors of each species can also be included in the phenotypic differentiation, such as the API 32C, the Auxacolor system (Campbell *et al.*, 1998), BactiCard *Candida* and Murex *C. albicans* (Crist *et al.*, 1996). These tests are more expedite, simple and as accurate as the ones described before.

The most expedite and less expensive method commonly used to differentiate *Candida albicans* and *Candida dubliniensis* is the CHROMagar *Candida* (Beighton *et al.*, 1995; Jabra-Rizk *et al.*, 2001a), this solid medium allows a differentiation between several *Candida* species through the colour of the colonies formed (Figure 1.3)

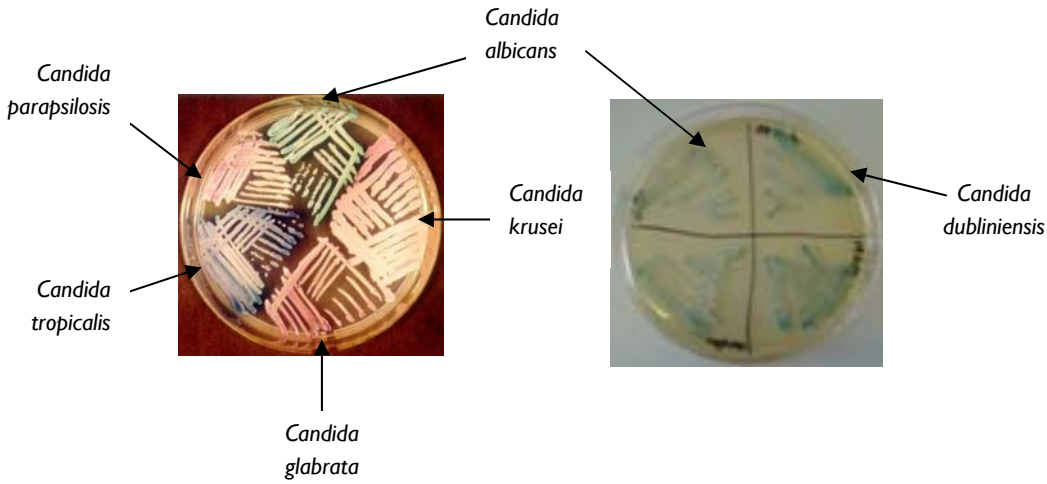


Figure 1.3 – Images of Petri plates with CHROMagar Candida with different *Candida* species.

Following 48 h of growth at 37 °C on CHROMagar Candida, *C. albicans* colonies appear light bleu-green, while *C. dubliniensis* colonies appear dark green, being easily distinguished (Coleman *et al.*, 1997; Sullivan and Coleman, 1998).

1.2 – Virulence factors

Like other pathogens, virulence in *Candida albicans* includes all the factors involved in the interactions with host, such as binding of the organism to host cells proteins or microbial competitors (co-aggregation) more than likely prevents or at least reduces the extent of clearance by the host. Additionally, several degradative enzymes have been shown to promote virulence. Invasion should be facilitated by the transition between yeast cells and filamentous growth (Calderone and Fonzi, 2001). Among the virulence factors can be highlighted the cell wall composition, the adhesion phenomenon, the biofilm formation and proteinases production.

1.2.1 – Cell wall

The cell envelope can be defined as the plasma membrane, the periplasmatic space, the cell wall and the fibrous layer associated with the outer region of the wall. The plasma membrane forms a permeable barrier between the cytosol of the cell and the external

environment. The periplasmatic space is the region bounded by the cell wall and the plasma membrane, including the space created by membrane evagination (Prasad, 1991).

The cell wall is essential both to the biology of the yeast and to its interactions with the human host in health and disease. This structure is responsible for supplying the rigidity that maintains the unique shapes that characterize fungal growth. The surface of the organism is the site of the physical interactions between the fungus and host proteins and tissues that lead to adherence and between fungus and the immune system that lead to clearance (Cannon and Chaffin, 1999).

The cell wall of *Candida albicans* (Figure 1.4) is composed of 80 to 90% of carbohydrates, 6 to 25% of proteins and 1 to 7% of lipids. The carbohydrates include branched polymers of glucose (β-glucans), unbranched polymers of *N*-acetyl-D-glucosamine (chitin) and polymers of mannose (mannan), covalently associated with proteins (Chaffin *et al.*, 1998).

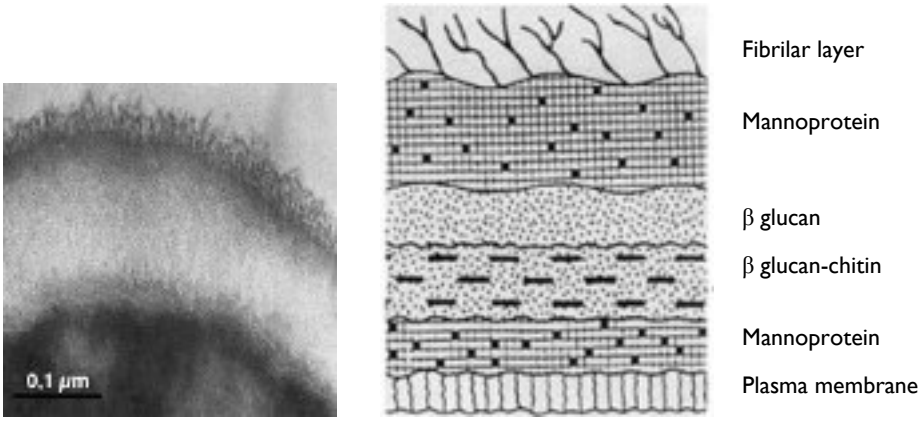


Figure 1.4 – SEM image (a) and schematic representation (b) of *Candida albicans* cell wall. Adapted from http://www.reviberoammicol.com/photo_gallery/Candida/albicans (2004).

The matrix polymers of *C. albicans* cell wall are basically mannoproteins, comprising also approximately 40% of the total cell wall polysaccharide; some molecules are directly involved in the architecture of the wall with nonenzymatic activity, while others have enzyme activities (Prasad, 1991). Mannan was first identified as the major cell surface antigen of *C. albicans* before the chemical structure was understood (Calderone and Braun, 1991; Summers *et al.*, 1964). Mannan by itself does not exist on the surface of the cell but is covalently bound to proteins as either a N-glycosidic bond through two di-N-acetylchitobiose (GlcNAc) units to an asparagine residue of a wall

protein or O-linked to either serine or a threonine residue of a wall protein (Chauhan *et al.*, 2002). Summers *et al.* (2002) revealed that mannan, rather than β-glucan or chitin, contributes to the serological specificity of each *Candida* spp. (Suzuki, 2002).

1.2.2 – Proteinase production

Proteins that are found in the in vitro growth medium are often called secreted or extracellular proteins. To reach this location, these proteins travel through the cell wall, where they coexist with cell wall-bound moieties and due to their location are proteins that contribute to the total cell wall proteinaceous component (Chaffin *et al.*, 1998). So, a number of hydrolytic enzymes can be recovered from cell wall and periplasm (enzymes that are thought to be involved in the cell wall biosynthesis) or from the culture medium (enzymes that are associated with the remodelling that accompanies growth and division of cells) (Chaffin *et al.*, 1998). The first case can include enzymes as exo-β-(1,3)-glucanase, β-1,3-glucan transferase, chitinase, β-*N*-acetylglucosaminidase and transglutaminase. The action of hydrolytic enzymes and proteins with extracellular targets, corresponding to the second group of enzymes, may provide access to nutrients for the organism. When hydrolysis of these substrates or action of extracellular proteins affect the function and viability of the host, the enzymes may be considered virulence factors that contribute to the establishment of infection. One of these enzymes is the *Candida*l secreted aspartyl proteinase (SAP), belonging to the group of acid proteinases, that was first identified by Staib (1969). Over 25 years the biological characteristics of these enzymes, including their role as a potential virulence factor of *C. albicans*, have been studied by several authors (Calderone and Fonzi, 2001; Naglik *et al.*, 1999; Penha *et al.*, 2000). Besides SAP, phospholipase, esterase, glucoamylase, hemolytic factor, acid phosphatase, lipase, hyaluronidase, chondroitan sulfatase, metallopeptidase and trehalase are also included in this group of enzymes (Chaffin *et al.*, 1998).

1.2.3 – Dimorphism

Candida albicans undergoes reversible morphological transitions between ovoid, unicellular budding cells (yeast cells or blastopores) and chains of filamentous cells (Figure 1.5). The latter cell morphism displays different degrees of filamentation, ranging from slightly elongated ovoid cells to significantly extended tube-like cells. Filamentous cells are classified either as pseudohyphae or hyphae depending on their morphology. Although the degree of elongation of pseudohyphal cells can vary considerably, from relatively short to significant extended cells, they always display constrictions at their septa between individual cellular compartments. In contrast, true hyphae and their progenitors (germ tubes) show no constrictions, having parallel walls at their septa (Calderone, 2002a).

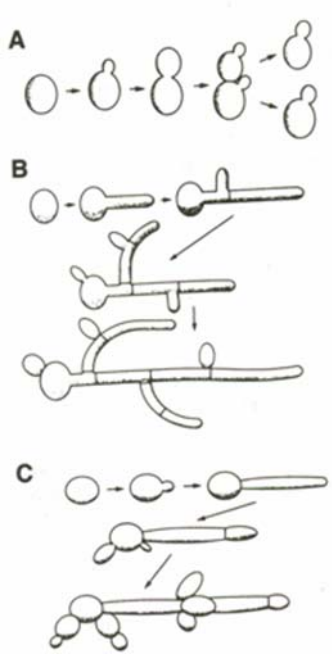


Figure 1.5 – Growth forms of *Candida* species: (A) yeasts, (B) hyphae and (C) pseudohyphae. Adapted from Calderone (2002a).

The contribution of dimorphic growth to virulence has been investigated in studies in which virulence was determined for mutants that can grow only in either the yeast or filamentous form (Prasad, 1991). Although virulence is decreased for such mutants, the strains used in those studies were produced by classical genetic methods and are likely to carry multiple genetic lesions (Prasad, 1991).

There are evidences that suggest that yeast-hypha morphogenesis is coregulated with other virulence factors. For instances, the SAP4-6 genes, which are the members of a large family of secreted aspartyl proteinase genes, that promote the virulence of *C. albicans* (Brown, 2002a; Hube *et al.*, 1997), are expressed specifically during hyphal development.

1.2.4 – Adhesion and biofilm formation

In order to proliferate in the oral cavity, yeast cells must adhere to the oral surfaces otherwise they are washed out by the salivary flows. So, one of the most important factors of virulence of *Candida* species is their ability to adhere using a variety of mechanisms, permitting the yeast to anchor at a site and the process of tissue colonization to commence (Cotter and Kavanagh, 2000).

Adherence to host tissue is achieved by a combination of specific and non-specific interactions.

The components of the organism that promote host recognition and colonization are referred to as adhesins. The adhesins of *Candida albicans* are usually of polysaccharide or glycoprotein nature (Calderone and Gow, 2002) and are also called binding proteins or receptors. Examples of adhesins are chitin, factor 6 oligomannosaccharide, 66-kDa fimbrial protein, fibronectin binding protein, iC3b binding protein, fucose binding protein, GlcNAc or glucosamine, SAP and ALS gene family (Cannon and Chaffin, 1999). So, *C. albicans* possesses multiple adhesins and there may be more than one adhesin that recognizes a host ligand or cell.

Included in the non-specific interactions, the mechanisms involved in the reversible adherence process, are the electrostatic interactions and the cell surface hydrophobicity (CSH) (Cotter and Kavanagh, 2000).

Adhesion in the oral cavity can occur to different surfaces as buccal epithelial cells (BEC), inert polymers and teeth. Adherence to BEC is affected by many host factors, and hormonal effects on adherence can be mediated by altering the expression of adhesins on *C. albicans* cells or ligands on host cells. Fibronectin was one of the first molecules to be suggested as a ligand recognized by *C. albicans* adhesin (Skerl *et al.*, 1984). The complement fragment iC3b has also been implicated as a ligand involved in epithelial and endothelial cell adherence (Gustafson *et al.*, 1991). SAPs are another

factor that appears to contribute to adhesion of *Candida albicans* to BEC and other substrates (Cannon and Chaffin, 1999; Watts *et al.*, 1998).

As prosthetic devices are among the major responsible for oral candidiasis, the adhesion of *Candida* species to polymeric materials has been one of the main goals of *Candida* studies (Edgerton *et al.*, 1993; Egusa *et al.*, 2000; Millsap *et al.*, 1999b; Nair and Samaranayke, 1996a; Nikawa *et al.*, 2001b; Nikawa *et al.*, 2001a; Radford *et al.*, 1998; Samaranayke *et al.*, 1980; Samaranayke and MacFarlane, 1980; San Millan *et al.*, 2000; Waters *et al.*, 1997). It is interesting to notice that all these studies are mainly focused on *Candida albicans* strains.

Adhesion to tooth surface, is the less studied factor, once it is the material more difficult to represent.

Considering the adhesion to inert polymers and teeth, this phenomenon is mainly ruled by CSH and electrostatic interactions. Electrostatic interactions are stronger but act over longer distances (>10 nm) than hydrophobic interactions, and are thought not to contribute to adherence in a major way (Cotter and Kavanagh, 2000).

Although adhesion is the first step in the infection process the biofilm formation is the subsequent step (Figure 1.6).

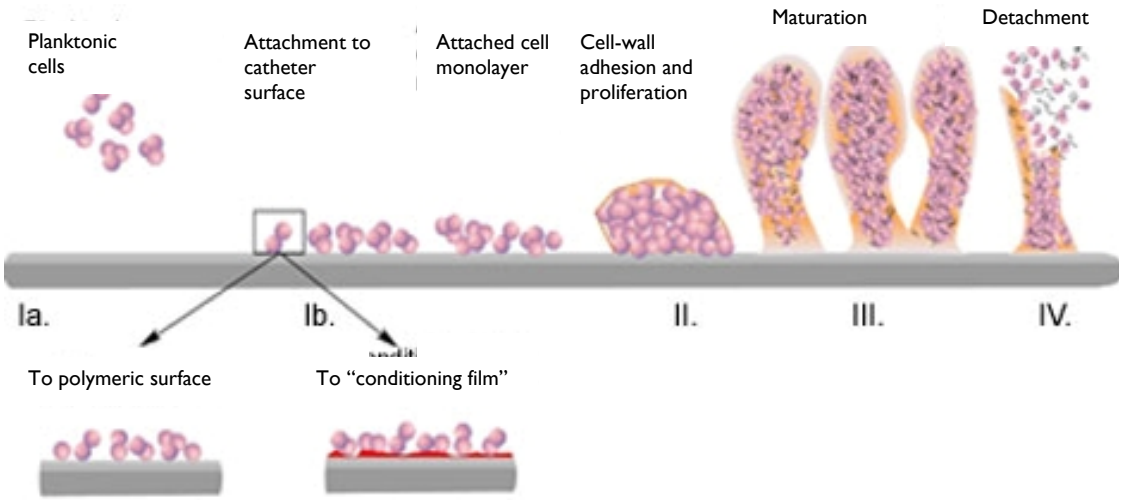


Figure 1.6 – Schematic biofilm formation on a polymeric surface. Cells in the planktonic form (Ia) get close to a surface, attach to it and form a cell monolayer (Ib). After that cells proliferate and produce extracellular polymers forming a biofilm (II). After some time the biofilm achieves maturation (III) and at the end cells start to detach from it (III). Adapted from http://www.niaid.nih.gov/dir/labs/images/ottofig.jpg (2004).

The biofilm formation includes adhesion of planktonic cells to the surface, growth and secretion of extracellular polymers (forming the mature biofilm) and cell detachment. Comparing the extracellular polymers (EP) of bacterial and yeast biofilms, the EP of yeast biofilm have significantly less amount of total carbohydrates and proteins, but higher proportion of glucose, also containing galactose, suggesting that the composition of the biofilm EP might be unique. In contrast to the extensive literature describing bacterial biofilms, little attention has been paid to medically relevant fungal biofilms. Two consequences of biofilm growth with profound clinical implications are the markedly enhanced resistance to microbial agents and protection from host defences (Ramage *et al.*, 2001).

The acquired resistance to antifungal agents has been one of the major problems of *Candida* infections once it allows the development of new strains that are resistant to these agents. Acquisition of resistance to azole compounds has been recorded with several organisms, in particular *C. albicans*, in situations where the drugs have been given for long periods of time in the face of persistent infection. Although this is not a new problem, it appears to be increasing in prevalence and importance (Warnock and Johnson, 1997).

There are different classes of antifungal agents including the azoles, the polyenes, allylamines or morpholine derivatives. These antifungal agents are designed based on the fact that ergosterol biosynthesis is specific to fungi and is necessary for their growth. One of the most successful polyene derivatives is amphotericin B. This antifungal agent can form soluble salts in both basic and acid environments, it is not orally nor intramuscularly absorbed and is insoluble in water. Within the group of azoles there are N-1 substituted imidazoles (ketaconazole, miconazole, clotrimazole) and triazoles (fluconazole, itraconazole).

The use of fluconazole, a drug that gained considerable importance as an alternative to amphotericin B, has resulted in the emergence of some *Candida* species as pathogens that are either inherently resistant to fluconazole or developed resistance to it.

1.3 – Oral cavity

Oral candidiasis is a significant problem in patients undergoing treatment for cancer or organ transplantation and is one of the most common and persistent conditions encountered in individuals with human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS) (Jacobs and Nall, 1997). The term oral candidiasis embraces a number of distinct clinical oral pathologies that include acute pseudomembranous candidiasis, erythematous candidiasis, chronic hyperplastic candidiasis, chronic atrophic candidiasis and angular cheilitis (O'Sullivan *et al.*, 2000).

The mouth is not a homogeneous environment for microbial colonization. Distinct habitats exist, as mucosal surfaces (lips, cheek, palate or tongue) and teeth which, because of their biological features, support the growth of a distinctive microbial community (Marsh, 2000). Sometimes there are also oral prosthetic devices, either made of polymers or metal ligands. Saliva is another constituent of the mouth that influences in a greater extent the development of microorganisms. So, the oral cavity presents a habitat that is paradoxically at once inviting and challenging to potential microbial colonization (Figure 1.7). A warm, moist, generally nutrient rich environment is a situation that would enhance colonization. Conversely, the mechanical shearing forces of salivary flow and tongue movement would tend to dislodge and expel microorganisms (Marsh, 2000).

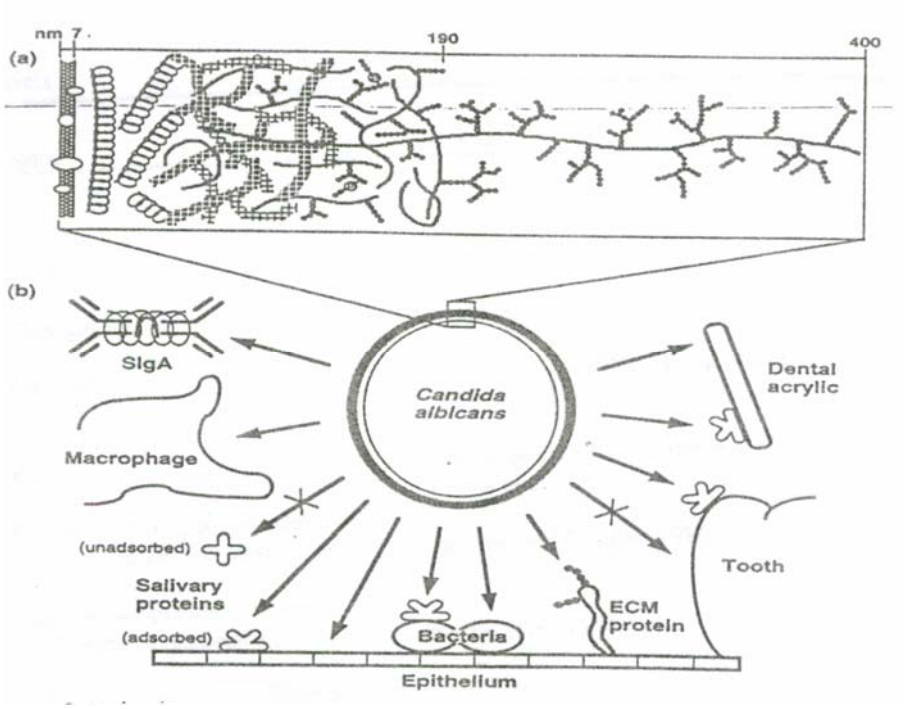


Figure 1.7 – Schematic representation of the different interactions that happen in the oral cavity. Adapted from Cannon and Chaffin (1999).

The growth in the oral cavity, can be influenced by several factors as temperature, redox potential/anaerobiosis, pH, nutrients (sugars, amino acids and peptides) or host genetics and social behaviour (Marsh, 2000).

1.3.1 – Teeth

The simplest sites to consider in oral adhesion are the centre-plane surfaces of the front teeth. Organisms will be carried to this position by the saliva which will then drain away to the extremities of the tooth surface (Ellewood *et al.*, 1979). The tooth is composed mainly by dentin and covered by an enamel layer that protects it (Figure 1.8).

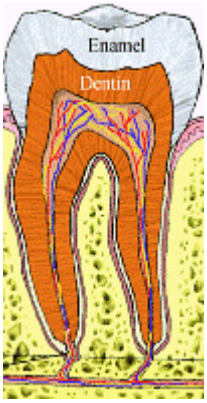


Figure 1.8 – Schematic tooth composition. Adapted from (http://medic.med.uth.tmc.edu/Lecture/Main/tool2.htm (2004).

A large proportion of the inorganic portion of dental enamel consists of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). So, hydroxyapatite (HAP) has been useful as a model for the study of dental plaque formation (Berry and Siragusa, 1997; Cowan *et al.*, 1987; Weerkamp and McBride, 1980), but these studies are related to bacteria not to yeasts. Regarding yeast adhesion to HAP, few studies have been reported (Berry and Siragusa, 1997; Clark *et al.*, 1978) and all using spherical beads of HAP.

1.3.2 – Dentures

Denture stomatitis is one of the most important types of *Candida* infections. The yeasts responsible for these infections are commonly found on the palatal surfaces of denture wearers (Budtz-Jorgensen, 1972; Davenport, 1970) and it is believed that the upper denture acts as a reservoir of infection (Nair and Samaranayke, 1996a). The prosthetic devices can include many different types of appliances, either fixed or removable, that are used to help move teeth, retrain muscles and affect the growth of the jaws. These devices can be made of polymeric materials, metallic frameworks or a combination of both (Figure 1.9).

Figure 1.9 – Examples of dentures with acrylic and metal alloys. Adapted from http://www.doctorspiller.com/Partial_Dentures.htm (2004).

The metallic framework is usually made of cast metal, which can be gold based or a chrome cobalt alloy. The polymeric materials can be acrylic or silicone rubber, either heat or room temperature cured.

1.3.3 – Saliva

The mean pH of saliva is between 6.75 and 7.25, which favours the growth of many microorganisms and the ionic composition of saliva promote buffering properties and has the ability to remineralise enamel (Marsh, 2000). Almost all studies of adhesion concerning the oral cavity use natural whole saliva (Edgerton *et al.*, 1993; Nikawa *et al.*, 1998; Samaranayke *et al.*, 1980). But its properties can vary with the donor and with the time of the day and so exact duplication is not possible. Thus, for in vitro testing, in order to replicate the results, artificial saliva is a good substitute. When artificial saliva is prepared there are some points to be considered as the pH and the buffer effect, CO_2 role (the pH should be adjusted by bubbling CO_2), ionic strength (approximately 0.043 mol l^{-1}) and specific conductivity, the presence of calcium ions, hydrogenophosphate and hydrogenocarbonate as well as tiocyanate ions (Gal *et al.*, 2001).

1.3.4 – Yeast/Bacteria interactions

The oral cavity is an ecological niche for different types of microorganisms, including yeast and bacteria. In vitro research (Nair and Samaranayke, 1996a; Verran and

Motteram, 1987) has demonstrated that *C. albicans* adhesion to acrylic surfaces is influenced by the presence of adhering *Streptococci*, such as *Streptococcus sanguis* and *Streptococcus salivaris*, suggesting a potential role for adhesive interactions between yeasts and bacteria in their colonization of denture surfaces (Millsap *et al.*, 1998). Adhesive interactions between yeasts and bacteria have not been extensively studied when compared with adhesive interactions between bacteria. The colonization of hard tissues surfaces in the oral cavity, for example, occurs in an ordered succession between initially colonizing strains, such as *S. sanguis* and *Actinomyces naeslundii*, and later colonizers (Millsap *et al.*, 1998).

1.4 – Scope of the work

In order to contribute to the understanding of the phenomena that involves *Candida* infection, the main goal of this work was the comparison of the behaviour of two *Candida* species, *Candida albicans* and *Candida dubliniensis*. So, in order to accomplish this goal the physico-chemical surface properties of the yeast cells were studied, as well as the adhesion phenomenon, either to inert surfaces and epithelial cells, the biofilm formation and the effect of subinhibitory concentrations of antifungal agents in both adhesion and biofilm formation.

1.5 – Structure of the dissertation

This dissertation is divided in 6 chapters. The first one includes a general introduction to the work presented here and also the state of the art. The methods used, the results obtained and its discussion are separate in different chapters, each one corresponding to a part of the experimental work done. Chapter two concerns the studies of yeast cells adhesion to inert surfaces, while in chapter three is described yeast adhesion to epithelial cells. Chapter four includes the biofilm studies and the methods used to their evaluation. The influence of subinhibitory concentrations of the antifungal agents is presented in chapter five. The last chapter regards the main conclusions and the suggestions for future work.

Chapter 2 – ADHESION TO INERT SURFACES

2.1 – Introduction

Colonization of the oral cavity by yeast cells is a complex phenomenon involving adhesion to epithelium, teeth and inert buccal materials used as prostheses or implants.

2.1.1 – Adhesion phenomenon

Since the oral cavity is a continuous-flow environment, yeast cells present in the oral cavity are washed out by saliva and swallowed unless they adhere and replicate. Moreover, growth conditions in the oral cavity are so poor that cells have to adhere to be maintained (Cannon and Chaffin, 1999; McCullough *et al.*, 1996). So, an essential pre-requisite for the successful colonization of host tissues by these microorganisms is the ability to adhere to superficial epithelial cells, or to a closely associated surface (Samaranayke and MacFarlane, 1980). Denture stomatitis is common among denture wearers. Numerous yeasts are commonly found on the palatal surface of the denture (Pfaller *et al.*, 1996) and this supports the theory that the upper denture acts as a reservoir for infection. Thus, it is of major importance the study of the process of adhesion of *Candida* spp. to oral surfaces, such as prosthetic devices (polymers) and teeth enamel, which is mainly composed of calcium phosphate, usually simulated by hydroxyapatite (HAP).

Adhesion of microorganisms to a substratum can be ruled by several mechanisms and several conditions should be met, as cell surfaces and substratum must have matching properties, an adequate aqueous environment must be provided, hydrodynamic conditions must be favourable and sufficient time must be allowed (Doyle, 1991). Figure 2.1 presents the different steps that lead to firm adhesion.

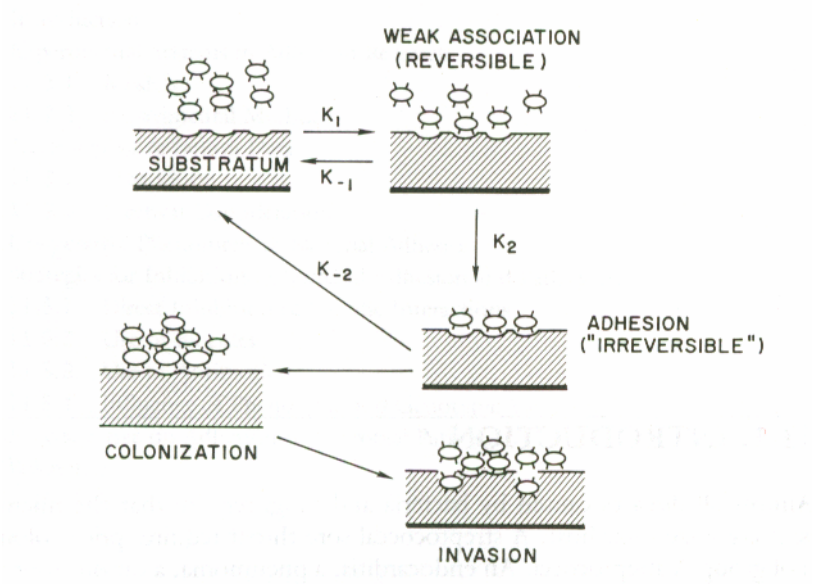


Figure 2.1– Steps involved in adhesion of microorganisms to inert substratum. Adapted from Doyle (1991).

The adhesion process begins with the approach of microorganisms to the substratum and a loose bounding with only few reversible interactions (K_1 rate). The microorganism ligands may subsequently combine with complementary receptors, creating an irreversible adhesion (K_2 rate). The rate constant K_{-2} is very low, showing that dissociation is a critical step in the formation of a stable cell-substratum union. Once firmly adherent, the microorganism may divide (colonize) and may produce symptoms of disease (invasion).

During the adhesion process several levels of energy are involved as it represented in Figure 2.2.

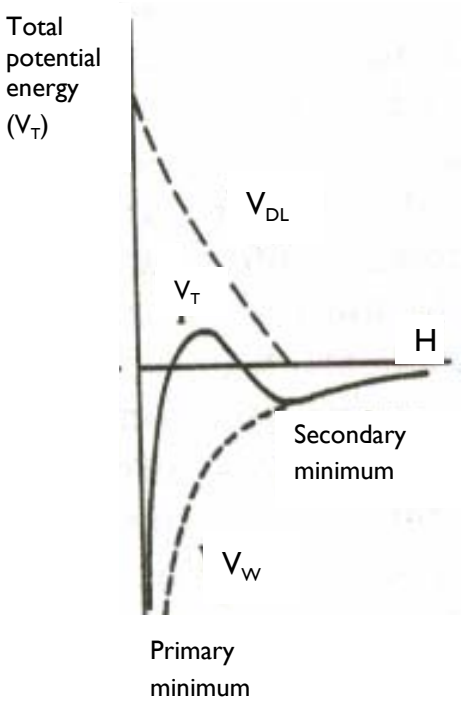


Figure 2.2 – Total potential energy of interaction (V_T) as a function of separation distance (H), represented as the sum of attractive van der Waals interactions (V_W) and repulsive electrical double layer interactions (V_{DL}). The secondary minimum corresponds to reversible adhesion and the primary minimum to irreversible adhesion. Adapted from Oliveira (1997).

According to Marshall (1991) van der Waals forces are the only ones operative at separation distances greater than approximately 50 nm. This distance is considered to be too large for the opposing surfaces to recognize specific surface components. At separation distances between approximately 10 and 20 nm, secondary minimum interactions occur as a result of van der Waals and electrical double layer forces and adhesion is probably reversible. Hydrophobic groups on the cell and substratum surfaces at this point may play a major role in removing water films from between the interacting surfaces enabling short-range interactions to occur. At the same time, the microorganisms may show some physiological response to the presence of the substratum in the form of modification of the surface structure of the cell. At separation distances of less than about 1.5 nm, where any potential energy barrier has been overcome, a variety of specific or nonspecific short-range forces can occur that lead to irreversible adhesion (Marshall, 1991). The surface properties that can rule the adhesion phenomenon are hydrophobicity, charge, roughness and chemical composition.

Hydrophobicity

According to van Oss and Giese (1995) in biological systems hydrophobic interactions are usually the strongest of all range of the non-covalent interactions. Albeit the importance of hydrophobicity in the adhesion process it has been very difficult to establish an universal definition of hydrophobicity, especially because different experimental methodologies have been used for its measurement. In the case of microbial cells there is a wide variety of methods like: microbial adhesion to hydrocarbons - MATH, such as xylene (Samaranayke *et al.*, 1995) and hexadecane (Rodrigues *et al.*, 1999); salt aggregation (Lindhal *et al.*, 1981); hydrophobic interaction chromatography (Hjerten *et al.*, 1974); hydrophobic microsphere assay - HMA (Hazen *et al.*, 2001; Hazen and Hazen, 1987); co-aggregation with *Fusobacterium nucleatum* – CoAg (Jabra-Rizk *et al.*, 2001c) and contact angles (van der Mei *et al.*, 1998). The most common methods used to determine *Candida albicans* hydrophobicity are MATH and HMA, but these methods only give qualitative information, because they are based on the microorganism affinity for a polar and an apolar phase or a ligand. The problem with these methods, and others like MATH, is that other forces (as electrostatic), besides hydrophobic ones, may interfere in the interaction between the ligands and the cell surface and so, surface hydrophobicity is being masked. Doyle (2000) analysed different methods to determine hydrophobicity and concluded that the contact angle method is probably the most definitive way to determine cell surface hydrophobicity, once it gives an average degree of hydrophobicity and does not take into account cell cycle variations or individual cell contributions. According to this method, hydrophobicity is expressed in terms of the contact angle formed by a sessile drop of water on a lawn of microbial cells. The hydratation of the surface is the major problem of this method, which can be overcome preparing an adequate cell lawn.

The relation between the contact angle (θ) formed by a liquid over a solid surface and the components of the surface tension (of the liquid-l and surface-s): apolar (γ^{LW}: Lifshitz-van der Waals) and polar ($\gamma^{AB} = 2 \times \sqrt{\gamma^{+}\gamma^{-}}$: Lewis acid-base) can be established by the Young-Good-Girifalco-Fowkes equation (van Oss *et al.*, 1987).

$$(1 - \cos\theta)\gamma_l = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^{+}\gamma_l^{-}} + \sqrt{\gamma_s^{-}\gamma_l^{+}}\right) \quad \text{Eq. 2.1}$$

As this equation has three unknown surface parameters, three different liquids (two polar and one apolar) are needed to calculate the surface tension ($\gamma^{tot} = \gamma^{AB} + \gamma^{LW}$). It

is important that the probe liquids have a surface tension higher than that of the solid sample to avoid the spreading of the liquid on the surface. So, this parameter should be higher than 40 mJ m^{-2} (van Oss *et al.*, 1987). Between the polar componets, water and formamide are the most commonly used and α-bromonaphthalene and di-iodometane are the apolar ones. According to van Oss and Giese (1995) the hydrophobicity of a given material (s) can be defined in terms of the variation of the free energy of interaction between two moieties of that material immersed in water (w). The free energy comprises a polar (AB) and an apolar (LW) component ($\Delta G_{sws}^{tot} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB}$) and the variation of the total free energy is given by:

$$\Delta G_{sws}^{tot} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 - 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \qquad \text{Eq 2.2}$$

When the value of ΔG_{sws}^{tot} is negative (the free energy of interaction between molecules is attractive) it means that the cells have less affinity for water than among themselves, meaning that they have a hydrophobic character. On the contrary, cells are hydrophilic when this value is positive (ΔG_{sws}^{tot} >0).

Electron microscopy demonstrated the presence of mannoprotein fibrils on the surface of both hydrophilic and hydrophobic cells, the length and distribution of which varied between the two cells types (Masuoka and Hazen, 1997). Both hydrophobic and hydrophilic cells possess similar hydrophobic proteins, but the amounts of those proteins at the surface are dramatically different (Hazen and Hazen, 1992). The CSH of yeast cells is affected by various factors, including temperature, nutrition and stage of growth (Fukuzawa and Kagaya, 1997).

Surface charge

Microbial cell surface, when in aqueous medium, as all liquid-solid and liquid-gas interfaces, possesses an electric charge; that is usually negative. The surface charge is a result of the ionization of the surface molecules or adsorption of ions from the liquid phase. Such charge can be evaluated by a variety of techniques, e.g., titration, ion-exchange chromatography, surface conductivity and electrokinetics (James, 1991). The latter is one of the most common methods to access surface charge, through the measurement of electrophoretic mobility and the calculation of zeta potential. In this

non-destructive method, the cells are studied in free suspension and so any information obtained refers to cells in their natural hydrated state (James, 1991).

Chemical composition

Surface chemical composition determines several surface properties involved in adhesion such as hydrophobicity and charge. Additionally specific interactions are often mediated by certain surface molecules or groups. According to Rouxhet and Genet (1991) the relative properties of certain microbial cell surface molecules can be estimated by surface elemental composition determined by X-ray Photoelectron Microscopy (XPS). This technique involves irradiation of the sample by an X-ray beam, which induces ejection of photoelectrons. The kinetic energy of the emitted electrons is analyzed, and their binding energy in the atom of origin is determined. Due to inelastic scattering of electrons in the sample, the collected information concerns only the outermost molecular layers of the surface (2 to 5 nm) (Dufrêne *et al.*, 1997). As adhesion involves both inert surfaces and microbial cells, the study of both is of major importance. Inert surfaces have been characterized by XPS for a few years (Rouxhet *et al.*, 1994). But, on account of the complexity when working with microbial cell surfaces, the application of XPS appears to be more difficult. Indeed, culture conditions, specific nutrients and the physiological status of the organisms under study are an additional source of variation as compared with metal or polymer surfaces. Furthermore, necessary sample preparation steps as washing, centrifugation and freeze-drying may greatly affect the cell surface composition (van der Mei *et al.*, 2000). Nevertheless, XPS analysis has been improved by several authors (Boonarte and Rouxhet, 2000; Dufrêne *et al.*, 1997) in order to develop its applicability to cell surfaces.

Roughness

Roughness can be of significant importance not only by increasing the surface area, but also because it might include microbial establishment due to mechanical retention (Busscher and Evans, 1000). According to Verran *et al.* (1991), since the type and the degree of roughness of an inert surface clearly affect its colonization by microorganisms, the measurement of the roughness would be a valuable parameter to

include in studies of microbial adhesion to appropriate surfaces. Among the several constants that can be obtained after the roughness measurement the most commonly considered is the arithmetic mean of the departures of the profile from the mean line, known as Ra, being the value used in relation to microbial adhesion to hard surfaces. Studies on abutments of intraoral two-stage implants (Bollen *et al.*, 1996) indicated that an increase in the surface roughness (up to 0.8 μm) of these intraoral hard surfaces had a significant effect on the in vivo rate of plaque formation only if the initial surface had a minimum Ra value of 0.2 μm. Therefore, a "threshold Ra" was suggested, which can be located at a Ra score of 0.2 μm (Bollen *et al.*, 1997).

2.1.2 – Ways to access adhesion

The first approach to the in vitro adhesion studies includes static assays, either adhesion to the bottom of the wells of microtiter (ELISA) plates or to coupons representative of the surfaces. As in vivo adhesion always takes place in environments with different body flows, dynamic assays should be performed using either flow cells or bioreactors. Adhesion of *Candida* species to the oral cavity has been studied using both models, although the static methods are more used than the dynamic assays (Millsap *et al.*, 1999a).

Enumeration of adhered cells has been performed by several different methods, either direct or indirect. Included in the direct methods are the observations by light and fluorescence microscope, inverted microscope (Williams *et al.*, 1998) and scanning electron microscope (SEM) (Sen *et al.*, 1997). When the surfaces are transparent the light microscope can be used and the samples can be observed as they are or be stained with a contrast dye as crystal violet (Nair and Samaranayke, 1996a). Some surfaces are not translucent and need to be observed in fluorescent microscope with the help of fluorescent stains. These stains can die both live and dead cells, as acridinorange (Waters *et al.*, 1997) or calcofluor white (San Millan *et al.*, 2000), or dye specifically the live cells, as Syto nucleic acid stains (Raad *et al.*, 2003). The direct methods are only applicable when the surfaces do not present high roughness or do not have a flat side (as HAP beads). To overcome this problem, methods as ATP or CFU determination (Nikawa *et al.*, 1998) and radiollabelling (Edgerton *et al.*, 1993) can be used. The advantage of the direct methods is the accuracy that can be achieved,

once the enumeration of the cells is direct, but sometimes it turns important to have more expedite methods to allow a rapid determination of the number of adhered cells.

2.1.3 – Aims

The present chapter reports the study of the adhesion of two strains of *Candida albicans* and two strains of *Candida dubliniensis* to hydroxyapatite (simulating the tooth surface) and acrylic (commonly used as prosthetic material). In order to complement these studies the surface properties of both yeast cells and inert materials are determined, namely, surface tension parameters and degree of hydrophobicity, surface composition and electric charge.

2.2 – Materials and Methods

2.2.1 – Media

Cells were grown in Sabouraud dextrose broth (SDB - Merck), that is the most common medium used to grow *Candida* species. The adhesion assays were performed in ultrapure water and artificial saliva.

Sabouraud dextrose medium

The yeast cells were maintained in Sabouraud dextrose agar (SDA) that was prepared according to the manufacturer's instructions (30 g l^{-1}) plus the addition of agar, 1.7% (Merck). Sabouraud dextrose broth (SDB – Merck) was used as growth liquid medium and prepared using 30 g l^{-1} in water.

Artificial saliva

In some experiments artificial saliva was used to mimic the *in vivo* oral conditions. The artificial saliva was prepared according to Gal *et al.* (2001) with the following composition in mg l^{-1}: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5 KH_2PO_4, 200.0 Urea, 763.2 $Na_2SO_4.10H_2O$, 178.0 NH_4Cl, 227.8 $CaCl_2.2H_2O$ and 630.8 $NaHCO_3$. The pH was adjusted to 6.8 with carbon dioxide.

2.2.2 – Yeast cells

The *Candida* species studied were *Candida albicans* and *Candida dubliniensis* and two different strains of each species were used. In the case of *Candida albicans,* two clinical isolates were assayed: *Candida albicans* 12 A and *Candida albicans* 46B. The *Candida dubliniensis* cells were obtained from Centraalbureau voor Schimmelcultures (CBS): *Candida dubliniensis* 7987 and *Candida dubliniensis* 7988.

For all the assays, the yeast cells were first grown for 24 h in SDA at 37 °C. The cells were then inoculated in SDB for 18h at 37°C and 150 rpm. The selection of the period of incubation, 18h, was based on the work developed by other authors.

After incubation cells were harvested by centrifugation for 10 min at 5000 rpm and 4°C and washed twice with sterile ultrapure water.

Phosphate saline buffer (PBS) is a common medium (Nair and Samaranayke, 1996a; Samaranayke and MacFarlane, 1980; Waters *et al.*, 1997), in bacterial adhesion studies, but in the present case water was used since it does not lyse the yeast cells and do not form crystals as it occurs with PBS. The use of water as washing medium has also been reported by other authors (Nikawa *et al.*, 2001a). Besides being used as washing medium during centrifugation, water was used as control medium, against artificial saliva, in the adhesion assays.

2.2.3 – Oral surfaces

Two different materials were used as adhesion substrata: one to simulate the enamel of teeth - hydroxyapatite (batch P120 – Plasma Biotal; Tiedswell, UK) and the other is

commonly used in some prosthetic devices - self-polymerising acrylic (Vertex – Self Curing, Dentimex BV, The Netherlands).

The hydroxyapatite (HAP) discs were prepared with hydroxyapatite powder as reported by Lopes *et al.* (2000). The HAP powder was uniaxially pressed at 288 MPa in steel dies to produce 14 mm diameter discs using 4 g of powder. The discs were then placed on an alumina tile and fired to 1300°C at a rate of 4°C/min. The set temperature was maintained for 1 h, followed by natural cooling inside the furnace. After that, the discs were mechanically polished to 1 μm finish.

The acrylic coupons were prepared as described by Samaranayake and MacFarlane (1980). Briefly, 1.5 g of self-polymerizing acrylic powder was mixed with 1 ml of monomer liquid and after mixing the solution was poured onto a surface covered with aluminium foil. After 45 s another aluminium foil was placed on the top of the polymerizing mixture. The acrylic sheet, polymerized during 30 min, was cut into 8 × 8 mm^2.

2.2.5 – Adhesion assays

Coupons of acrylic and HAP were inserted in a 24 well plate and 2 ml of a cell suspension of 10^7 cells ml^{-1}, prepared with water or artificial saliva, were added to each well. After 1 h of incubation (100 rpm, at 37°C) each well was washed twice with ultrapure water, by removing carefully only the liquid above the coupon. Finally all the liquid was removed.

The amount of cells used was chosen according to previous studies (Nikawa *et al.*, 2001a; Radford *et al.*, 1998; Williams *et al.*, 1998) and the criteria was that this amount should be enough to be easily seen under the microscope but not too much to form aggregates that could not be counted. The selected number of cells in the initial suspension was 1×10^7 cells ml^{-1}.

To optimize the time for the adhesion assays of *Candida albicans* 12A and *Candida dubliniensis* 7987 to acrylic different times were assayed: 15, 30, 45, 60, 180 and 420

min. The amounts of adhered cells following these periods of time are presented in Figure 2.3.

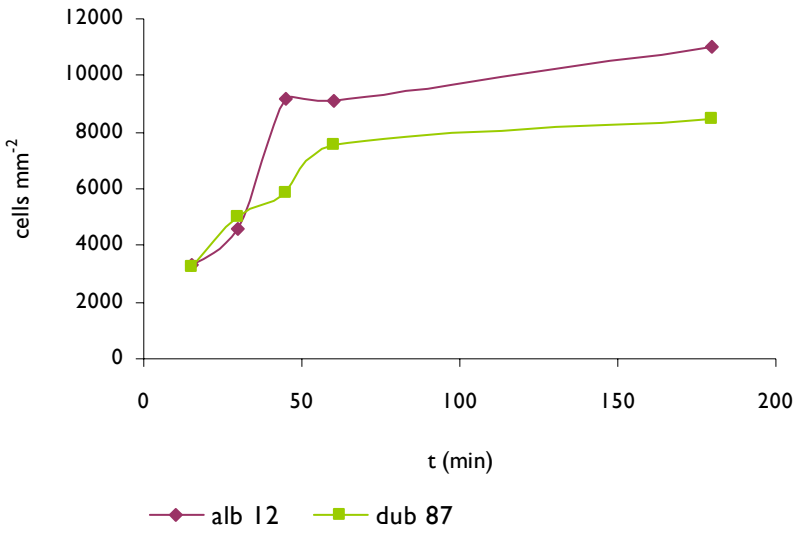


Figure 2.3 – Number of adhered cells of *Candida albicans* 12A (alb 12) and *Candida dubliniensis* 7987 (dub 87) obtained after 15, 30, 45, 60 and 180 of contact with acrylic.

Observing Figure 2.3 it is possible to see that after 60 minutes of contact the number of cells adhered to the surface reached a plateau.

Cell enumeration

After removing all the washing liquid from the wells, the coupons were stained with 500 µl of SYTO-13, 5µM in water (Molecular Probes) for 5 min, and observed under epifluorescence microscopy (450 - 490 nm). The images were captured with a video camera that was coupled to the microscope and connected to a computer. An image analysis system (SigmaScan Pro 5, SPSS) was used to quantify the adhered yeast cells. Twenty five fields were randomly counted in each sample. Each experiment was repeated six times.

2.2.4 – Surface properties

The surface properties of yeast cells and inert surfaces (acrylic and HAP) relevant for adhesion were characterized. This included the determination of surface tension and

surface free energy by contact angle measurements, zeta potential by electrophoretic measurement, surface composition by X-Ray Photoelectron Spectroscopy (XPS) and surface roughness with a laser profilometer.

Contact angle measurement

The cell lawns were prepared according to Henriques *et al.* (2002). A solution of 20 g l^{-1} of agar, and 10% (v/v) of glycerol was cast onto a microscope slide (75× 25 mm). An aliquot of 2 ml of 10^9cells ml^{-1} (prepared in water or saliva) was spread over the solidified agar layer, in order to cover the entire surface. This layer was let to dry at 37°C (for 10-15 h), and two more layers were added, with the same drying period between them.

Contact angles were measured on the yeast cell lawns or on the inert surfaces by the sessile drop technique using an apparatus model OCA 15 PLUS, DATAPHYSICS.

The measurements were performed at room temperature, using three different liquids: water, formamide and α-bromonaphthalene. Each assay was performed in triplicate and at least 10 contact angles, per sample, were measured.

The surface tension parameters were determined using a set of equations (Eq. 2.1 and 2.2) that were presented in the Introduction (section 2.1.1).

X-ray Photoelectron Spectroscopy (XPS)

A volume of 200 ml of an aqueous cell suspension (10^9cells ml^{-1}) was vacuum filtered through a nitrocellulose membrane of 45 μm. The membrane, completely covered with cells, was immediately frozen with liquid nitrogen and then stored at -80°C until the subsequent step of liophilization. Freeze drying was performed at 10 Pa, overnight. The samples were placed in a dessicator at room temperature and immediately analysed by XPS. The XPS analysis was performed using an apparatus ESCALAB 200A, with a VG5250 software and data analysis. The spectrometer used monochromatized Mg (Kα) X-ray radiation (15000 eV). The constant pass energy of the analyser was 20 eV and it was calibrated with reference to Ag $3d_{5/2}$ (368.27 eV). The pressure during analysis was under 1×10^{-6} Pa. The spectra for yeast cells were recorded following the sequence C_{1s}, O_{1s}, N_{1s}, P_{2p}. In the case of HAP and acrylic, the spectra sequence was: C_{1s}, O_{1s}, N_{1s}, P_{2p}. Si_{2p}, Na_{1s}, $Ca_{2p(2p3+2p1)}$, $K_{2p(2p3+2p1)}$.

Zeta potential

Cells were diluted in water and artificial saliva to an absorbance of approximately 0.1 (measured at 600 nm). The zeta potential was determined by electrophoretic mobility by means of a Zeta-Meter 3.0+.

Roughness

The surface roughness of acrylic and HAP was determined using a laser profilometer (Surftest SV 512) and the measurements were done in both longitudinal and transversal directions.

2.2.5 – Statistical analysis

The resulting data were statistically analysed using SPSS (Statistical Package for the Social Sciences). Two way ANOVA was used to perform multiple analysis of the interaction between all factors. One way ANOVA with Bonferroni test was used to compare the number of adhered cells of the four strains. All tests were performed with a confidence level of 95%.

2.3 – Results

The adhesion assays were performed using two strains of *Candida albicans* and of *Candida dubliniensis*, two materials, acrylic and HAP and two different media, water and artificial saliva. The corresponding results are presented in section 2.3.1. To help the explanation of those results the surface properties of both yeast cells and inert materials were determined and are present in sections 2.3.2 and 2.3.3, respectively.

2.3.1 – Adhesion results

The number of cells of *Candida albicans* and *Candida dubliniensis* adhered to each type of material immersed in water or in artificial saliva is presented in Table 2.1

Table 2.1 – Number of cells of *C. albicans* 12A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 adhered to acrylic and hydroxyapatite (HAP) in the presence of water and saliva

	Mean number of yeasts mm^{-2} (±SD[a])			
	Water		Saliva	
Strain	Acrylic	HAP	Acrylic	HAP
C. albicans 12A	4958 ± 887	18746 ± 6891	17916 ± 4492	15385 ± 1820
C. albicans 46B	8071 ± 3021	15903 ± 6669	16209 ± 1280	18894 ± 2958
C. dubliniensis 7987	6628 ± 2444	15887 ± 2018	14333 ± 644	18531 ± 2532
C. dubliniensis 7988	6342 ± 1875	12836 ± 3967	15065 ± 3130	19107 ± 3509

[a]SD, standard deviation

The Two-way ANOVA (Table 2.1) revealed that adhesion is mainly influenced by the type of material (HAP and acrylic) and the liquid medium where adhesion occurred (saliva or water) (p=0.000 for both cases). For each type of material, no statistical difference was found between the number of adhered cells of the strains studied (p=0.655).

To better understand the influence of each factor in the process of adhesion, a statistical analysis (One-way ANOVA) was performed with each variable, separately. When water was used as the adhesion medium, the differences in the number of cells adhered to HAP and to acrylic were significantly different (values of p were 0.001, 0.014, 0.000 and 0.008 for each strain, respectively as ordered in Table 2.1), with a higher number adhered to HAP than to acrylic. In the case of saliva, these differences were not significant (values of p for the strains *C. albicans* 12A, *C. albicans* 46B and *C. dubliniensis* 7988 were 0.277, 0.107 and 0.091, respectively), except in the case of *C. dubliniensis* 7987 (p=0.018). Considering the adhesion to acrylic, the number of cells attached in the presence of artificial saliva was significantly greater than in the presence of water (p=0.003 for *C. albicans* 12A and p=0.001 for the other strains). For HAP the results of the assays performed in water and saliva were not significantly different (p equal to 0.225, 0.308 and 0.086 for strains *C. albicans* 12A, *C. albicans* 46B

and *C. dubliniensis* 7987, respectively) except in the case of *C. dubliniensis* 7988 ($p=0.022$).

2.3.2 – Cell surface properties

The surface properties of microbial cells were studied considering different aspects as cell surface composition, hydrophobicity and charge.

The results obtained by XPS analysis on the C, O, N and P composition of the yeast cells surfaces were expressed in terms of ratios (Table 2.2). The proportion of proteins, polysaccharides and hydrocarbons in the composition of cell wall surfaces was calculated according to Rouxhet *et al.* (1994).

Table 2.2 – Ratios of O/C, N/C and N/P obtained by XPS analysis of the cell walls of *C. albicans* 12 A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988

Strain	O/C	N/C	N/P	Pr (%)	Ps (%)	Hc (%)
C. albicans 12A	0.61	0.09	5.37	30.6	65.3	4.1
C. albicans 46B	0.60	0.08	5.56	28.0	65.9	6.1
C. dubliniensis 7987	0.60	0.08	5.98	29.4	65.4	5.3
C. dubliniensis 7988	0.58	0.07	8.10	23.7	67.5	8.8

From Table 2.2 it can be observed that the ratios and the percentages of cell wall components are similar for all the strains studied.

In order to evaluate the influence of the liquid medium on surface properties, surface tension, hydrophobicity and zeta potential measurements were performed with cells conditioned with water or artificial saliva (Tables 2.3, 2.4 and 2.5). The degree of hydrophobicity of yeast cell surfaces (ΔG_{sws}) was expressed by the free energy of interaction between two identical surfaces (s) immersed in water (w), as proposed by van Oss and Giese (1995). ΔG_{sws} was determined with the contact angles of water, formamide and α-bromonaphtalene, after the calculation of γ^+ and γ^- (electron acceptor and electron donor parameters of the acid-base component of the surface tension) and γ^{LW} (the Lifhsitz van der Waals component of surface tension).

Table 2.3 – Values of contact angles measured with water (θ_w), formamide (θ_f) and α-bromonaphtalene (θ_b) on cells lawns of *C. albicans* 12A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 conditioned with water or artificial saliva

Medium	Cells	θ_w (°) (±SD[a])	θ_f (°) (±SD[a])	θ_b (°) (±SD[a])
Water	*C. albicans* 12A	16 ± 5	18 ± 1	65 ± 5
	C. albicans 46B	17 ± 4	19 ± 3	60 ± 5
	C. dubliniensis 7987	24 ± 2	28 ± 2	44 ± 3
	C. dubliniensis 7988	18 ± 2	18 ± 2	57 ± 2
Artificial Saliva	*C. albicans* 12A	15 ± 2	16 ± 1	63 ± 7
	C. albicans 46B	13 ± 1	16 ± 3	83 ± 1
	C. dubliniensis 7987	13 ± 2	14 ± 2	62 ± 5
	C. dubliniensis 7988	10 ± 1	12 ± 2	61 ± 5

[a]SD, Standard Deviation

Table 2.4 – Values of the components of surface tension (γ^+, γ^-, γ^{LW}) and degree of hydrophobicity (ΔG_{sws}) of cells (*C. albicans* 12A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988) conditioned with water or artificial saliva

Medium	Cells	γ^+ (mJ m^{-2}) (±SD[a])	γ^- (mJ m^{-2}) (±SD[a])	γ^{LW} (mJ m^{-2}) (±SD[a])	ΔG_{sws} (mJ m^{-2}) (±SD[a])
Water	*C. albicans* 12A	5 ± 1	49 ± 6	23 ± 3	21 ± 3
	C. albicans 46B	4 ± 1	51 ± 2	25 ± 2	24 ± 4
	C. dubliniensis 7987	3 ± 1	52 ± 2	30 ± 1	27 ± 2
	C. dubliniensis 7988	6 ± 1	50 ± 2	24 ± 2	20 ± 3
Artificial Saliva	*C. albicans* 12A	3 ± 0	49 ± 3	32 ± 1	24 ± 2
	C. albicans 46B	13 ± 1	51 ± 2	13 ± 0	10 ± 1
	C. dubliniensis 7987	6 ± 1	52 ± 0	24 ± 2	22 ± 2
	C. dubliniensis 7988	6 ± 2	53 ± 1	25 ± 3	23 ± 4

[a]SD, Standard Deviation

The four strains studied showed similar values of surface tension and a predominantly electron donancy (Table 2.4). In the presence of saliva, a slightly increase in the electron acceptor parameter (γ^+) was verified for all strains. As far as hydrophobicity is concerned, all *Candida* strains studied showed positive values of ΔG_{sws} in all conditions assayed and so, can be considered hydrophilic. This is corroborated by the value of water contact angle (Table 2.3) that is lower than 50°. The ΔG_{sws} values were very

similar, with the exception of *C. albicans* 46B, that exhibited a lower degree of hydrophilicity.

Table 2.5 shows the values of zeta potentials of the four *Candida* strains when immersed in water. Under these conditions they all displayed negative zeta potentials and the values did not differ statistically. When immersed in saliva all strains did not show any electrophoretic mobility at 200, 100 and 50 mV, which was expected due to the high ionic strength of the medium. Thus, their zeta potentials were considered nulls.

Table 2.5 – Average values of zeta potential of the yeast cells (*C. albicans* 12A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988) measured in water

Medium	Cells	ζ(mV) (±SD[a])
Water	*C. albicans* 12A	-14.5 ± 4.2
	C. albicans 46B	-8.3 ± 3.3
	C. dubliniensis 7987	-17.8 ± 3.7
	C. dubliniensis 7988	-9.6 ± 2.7

[a]SD, Standard Deviation

2.3.2 –Inert surface properties

As adhesion involves both cells and materials, the surface properties of the two studied oral materials were also determined namely surface tension and hydrophobicity (Table 2.6), surface elemental composition (Table 2.7) and roughness (Table 2.8).

Table 2.6 – Values of contact angles measured with water (θ_w), formamide (θ_f) and 1-bromonaphatelene (θ_b) on acrylic and HAP conditioned with water or artificial saliva

Medium	Surface	θ_w (°) (±SD[a])	θ_f (°) (±SD[a])	θ_b (°) (±SD[a])
Water	Acrylic	75 ± 3	57 ± 3	32 ± 9
	HAP	53 ± 4	57 ± 6	47 ± 3
Artificial saliva	Acrylic	64 ± 4	44 ± 3	36 ± 3
	HAP	23 ± 3	25 ±83	43 ± 2

[a]SD, Standard Deviation

Table 2.7 – Values of surface tension components (γ^+, γ^-, γ^{LW}) and degree of hydrophobicity (ΔG_{sws}) of acrylic and HAP conditioned with water or saliva

Medium	Surface	γ^+ (mJ m^{-2}) (±SD[a])	γ^- (mJ m^{-2}) (±SD[a])	γ^{LW} (mJ m^{-2}) (±SD[a])	ΔG_{sws} (mJ m^{-2}) (±SD[a])
Water	Acrylic	1 ± 0	5 ± 1	39 ± 3	-45 ± 0
	HAP	0 ± 0	39 ± 4	31 ± 2	25 ± 3
Artificial Saliva	Acrylic	1 ± 0	16 ± 3	36 ± 1	-26 ± 4
	HAP	1 ± 0	50 ± 3	33 ± 1	30 ± 3

[a]SD, Standard Deviation

The electron donor parameter increases in the presence of saliva for both materials. Although that increase is more notorious for acrylic, γ^- is always higher in HAP (Table 2.7).

According to the values of ΔG_{sws}, HAP can be considered hydrophilic whereas acrylic, is hydrophobic (ΔG_{sws}<0 in both media). It is interesting to observe Table 2.6 where the value of water contact angle in acrylic is always higher than 50°C, meaning that it has a hydrophilic character.

Table 2.8 – Percentage of the different chemical elements detected in the surface of acrylic and HAP, contacted with water or saliva, determined by XPS analysis

	Acrylic		HAP	
Compounds (%)	Water	Saliva	Water	Saliva
C 1s	75.75	72.80	23.20	14.05
O 1s	22.48	23.11	45.85	46.16
N 1s	0.76	1.02	0.30	0.39
P 2p	0.04	0.32	12.50	14.18
Si 2p	0.97	1.92	--	--
Na 1s	--	0.83	--	12.09
Ca 2p (2p3+2p1)	--	--	18.15	9.87
K 2p(2p3+2p1)	--	--	--	3.25

The chemical elemental composition of acrylic and HAP pre-conditioned with water or artificial saliva, obtained by XPS analysis (Table 2.8) elicit the conclusion that saliva altered the surface composition of both materials with special relevance for HAP.

The roughness values of acrylic and HAP measured in two cross directions are presented in Table 2.9.

Table 2.9 – Values of the roughness of acrylic and HAP measured in two directions (longitudinal and transversal)

Material	Measurement direction	Ra (μm) (±SD[a])
Acrylic	Longitudinal	0.25 ± 0.03
	Transversal	0.21 ± 0.03
HAP	Longitudinal	0.54 ± 0.04
	Transversal	0.43 ± 0.03

[a]SD, Standard Deviation

The results of Table 2.9 show that there is no difference between the data obtained by the longitudinal and the transversal measurements for each type of material. HAP exhibits a surface roughness about two times that of acrylic.

2.4 – Discussion

It is well known that cell surface hydrophobicity plays an important role in adhesion of *Candida* species. Hydrophobic cells bind more readily, to epithelial cells and plastics, than hydrophilic ones (Masuoka *et al.*, 1999). Both *Candida albicans* strains used in this study exhibited hydrophilic cell surfaces under the conditions assayed. The same was observed for the two strains of *Candida dubliniensis*. Moreover, there were no significant differences between *C. albicans* and *C. dubliniensis* hydrophobicity, either conditioned with water or artificial saliva (Table 2.4). Other authors (Hazen *et al.*, 2001) found differences in the hydrophobicity of some strains of *C. albicans* and *C. dubliniensis* when grown at 37°C. The results reported by these authors were based on the hydrophobic microspheres assay method. However, the drawback of this method and others like co-aggregation (Jabra-Rizk *et al.*, 1999) and microbial adhesion to hydrocarbons (Samaranayke *et al.*, 1995), is that other forces (as electrostatic), besides hydrophobic ones, may interfere in the interaction between the ligands and the cell surface, and so, surface hydrophobicity is masked (Aguedo, 2003; Doyle, 2000). Furthermore, the fact that the results obtained in the present study are consistent across two strains of each *Candida* species strongly suggests that these results are not an artefact of chance strain selection.

According to Rouxhet *et al.* (1994), XPS analysis can provide an indication of the amount of polysaccharides, proteins and hydrocarbons present at the cell surface of yeast strains, which can be determined from the ratios O/C, N/C and N/P. As all the strains present similar cell wall elemental compositions (Table 2.2), the polysaccharides, proteins and hydrocarbons content is similar and in average the percentages are 66.0 $\pm$ 1.0, 27.9 $\pm$ 3.0 and 6.1 $\pm$ 1.0, respectively. These values are in accordance with the values reported by Chaffin *et al.* (1998) for the cell wall composition of *Candida albicans*.
The XPS results corroborated the similarity of the values of the surface tension, hydrophobicity and zeta potential (Tables 2.4 and 2.5), among all strains.

The liquid medium where adhesion took place strongly influenced the results. In the presence of artificial saliva the number of cells adhered to acrylic, of the four strains

studied, was significantly greater compared to water (Table 2.1). The environmental conditions, such as pH, ionic strength, temperature and components in solution can influence cell surface characteristics (Flahaut *et al.*, 1998), e.g. surface charge and hydrophobicity (Hsu and Huang, 2002). Moreover, ionic strength determines the thickness of the electrical double layer which has a direct influence on electrostatic interactions established in the adhesion events (Oliveira, 1997). Thus, it is expected that artificial saliva, which has a high ionic strength, influences adhesion to oral surfaces, lowering the electrostatic repulsion. Furthermore, the adsorption of salivary components can also affect adhesion. It has been reported by several authors that adhesion of *Candida albicans* to oral surfaces is influenced by saliva (Busscher *et al.*, 1997; Millsap *et al.*, 1999b; O'Sullivan *et al.*, 2000; San Millan *et al.*, 2000). Saliva can enhance the adhesion to acrylic surfaces (Millsap *et al.*, 1999b) and polystyrene (San Millan *et al.*, 2000), whereas adhesion to silicone rubber can be discouraged in the presence of saliva (Busscher *et al.*, 1997). This fact has been attributed to the adsorption of different salivary components, including mucins (Edgerton *et al.*, 1993), other salivary proteins (Cannon *et al.*, 1995) and secretory IgA (San Millan *et al.*, 2000).
It must be stressed that the saliva formulation used in this study is a synthetic one without proteins. In almost all studies concerning adhesion of oral microorganisms to surfaces the saliva used is obtained from donors (Nikawa *et al.*, 2001a; Ueta *et al.*, 2000). Nevertheless, natural saliva varies according to the donor and the time of the day, thus exact duplications are impossible. Furthermore, natural saliva contains proteins such as mucin that can coat the oral surfaces influencing adhesion by specific interactions. So, artificial saliva was used in this study, in order to focus only the physico-chemical interactions.
In the present study the adsorption of phosphate ions to acrylic surfaces, indicated by XPS analysis (Table 2.8), caused an increase in the electron donancy of this material (Table 2.7). Moreover, the cells in the presence of artificial saliva exhibited a slightly increase in their electron acceptor groups (Table 2.4). Thus, the increase in the number of adhered cells to acrylic in the presence of artificial saliva can be explained by an increase in the interactions between the electron donor groups of acrylic and the electron acceptor groups of cells. In the case of HAP, artificial saliva caused an increase in the number of electron acceptor groups, perhaps due to the adsorption of sodium and potassium ions, as observed by XPS analysis (Table 2.8). Probably, in

opposition to acrylic, there was no such significant increase in the interactions between the electron acceptor groups of cells and electron donating groups of HAP, which can explain the similarity in the number of adhered cells.

The preferential adsorption of phosphate to acrylic and sodium and potassium to HAP can be explained by the fact that acrylic is more electron accepting than HAP and that HAP is more electron donating than acrylic. It has been suggested by other authors (Busscher *et al.*, 1997) that the different behaviours of cell attachment in the presence of artificial saliva may be related to the hydrophobicity of the substratum surfaces which may stimulate the adsorption of different salivary components. This hypothesis seems to apply in the present case.

It is curious to notice that the number of cells attached to HAP was greater than to acrylic in the presence of water, although HAP is less hydrophobic than acrylic. Furthermore, the adhesion to acrylic increased in the presence of artificial saliva and in this medium acrylic was less hydrophobic. This indicates that the hydrophobicity of the adhesion substratum played a minor role in the process of adhesion. So it is expected that other types of interactions rather than hydrophobic ones might be governing the phenomenon. The interactions established between the electron acceptor groups of the cells and the electron donating groups of the materials, as hypothesized before, may be determining the adhesion phenomenon.

Studies done by Bollen *et al.* (1997) showed that an increase in the surface roughness of resin strips above an Ra value of 2 μm resulted in a dramatic increase in bacterial colonization of these surfaces in comparison to smooth strips (Ra = 0.12 μm). Verran and Maryan (1997) demonstrated that surface roughness may also facilitate *Candida albicans* retention in silicon prostheses. Taylor *et al.* (1998) verified that an increase in surface roughness (0.15 to 3.53 μm) enhanced *Candida albicans* retention. Although the values of roughness of both materials are low (Ra < 0.5 μm) the roughness of HAP is higher than that of acrylic (Table 2.9), which can, in part, explain the higher amount of cells adhered to HAP in the absence of artificial saliva. When artificial saliva was present this effect might have been overcame by other interactions.

It should be noted that the acrylic used in the present work was a cold-cure variety of acrylic. However, most dentures are made of a heat-cured acrylic form of resin. Nevertheless, according to Davenport (1972) there is no difference in surface

roughness between the heat and cold-cure acrylic resin. So, it is expected that they have similar surface properties as they are made by the same chemical compounds.

As the different *Candida* strains showed similar cell wall compositions and cell surface physico-chemical properties they were expected to exhibit the same type of physico-chemical interaction with the adhesion substratum. Accordingly, all strains studied exhibited the same capability to adhere to both types of surfaces. These results proved the similarity of the phenotypic characteristics of *Candida albicans* and *Candida dubliniensis*, with respect to physico-chemical surface properties. The extent of adhesion of *Candida* strains to teeth (HAP) and prostheses (acrylic) is similar in the presence of artificial saliva, which may indicate that both surfaces are equally important reservoirs for *Candida*l infections.

Chapter 3 - ADHESION TO EPITHELIAL CELLS

3.1 – Introduction

The interaction between *Candida* species and mucosal cells is believed to be one of the critical initial events in the development of Candidiasis. Thus, studies on the adhesion process of yeast cells to mammalian cells are important to understand the phenomena involved in the interactions between yeasts and human tissue. One of the critical steps in these studies is the culture of mammalian cells, which requires some specific techniques, since these cells are very difficult to obtain and maintain.

3.1.1 – Mammalian cells culture

From its earliest beginnings in the 1940s cell culture has played a major role in medical science (Doyle and Griffiths, 2000). The field of animal cell biotechnology itself was born from the need to cultivate cells on an industrial scale. The ability to fuse cells from different species to form a hybrid cell led directly to the development of numerous hybridoma cell lines (Doyle and Griffiths, 2000).

Cell culture for medical research embraces a wide range of cell types and systems. A broad classification of primary culture includes the organ culture, primary explant culture and cell culture (Figure 3.1) (Robinson *et al.*, 1956).

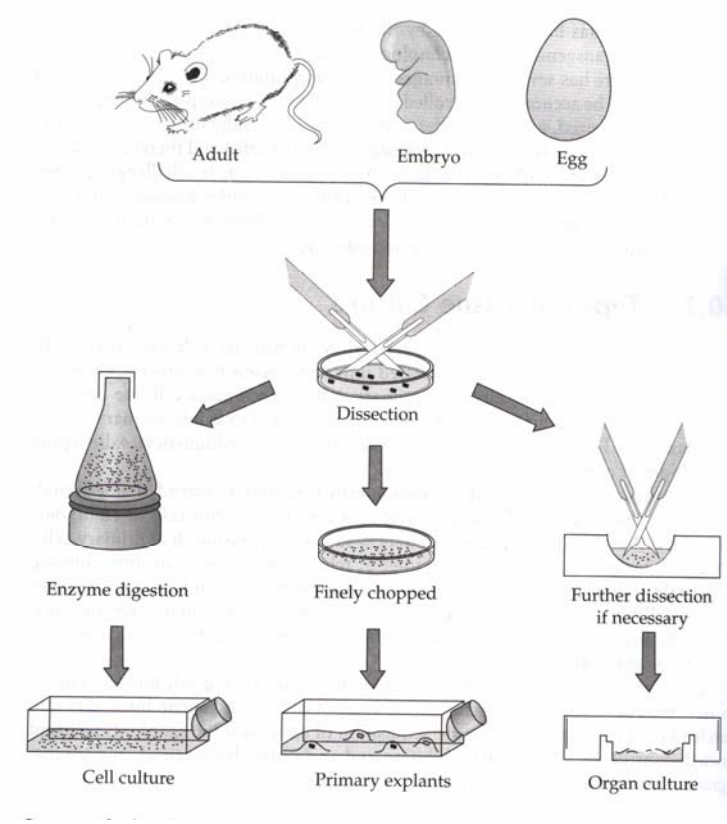


Figure 3.1 - Three main types of primary culture. Adapted from Palsson and Bathia (2004).

Primary explants

In the case of primary explants a fragment of tissue is placed at a liquid solid interface where, following attachment, outgrowth and migration of cells occur in the plane of solid substrate.

Organ culture

Considering the organ culture, the tissue explant retains, at least in part, its architectural features. It is placed in a culture environment that favours the retention of a three dimensional shape.

Cell culture

Cell cultures can be established from a wide variety of mammalian tissues by enzyme digestion at warm or cold temperatures (exfoliated) or by outgrowth from primary explants. Such cultures can be maintained in vitro for a limited period of time, the length of which is determined in part by whether or not cells are capable of

proliferation (Figure 3.2). It is possible, however, to obtain cultures of cells with unlimited growth potential.

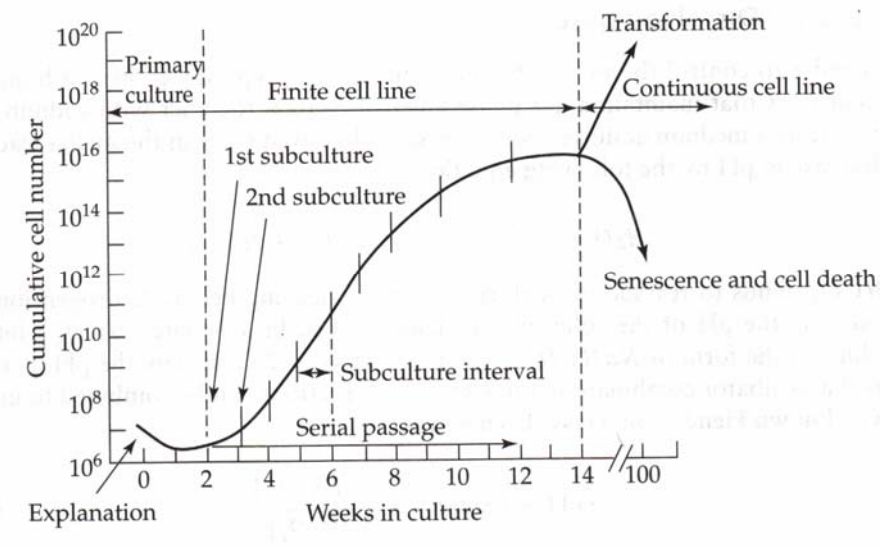


Figure 3.2 – Development of a cell line. Growth is relatively slow in the primary culture; growth enters a "log growth phase" where the growth curve is linear on a log scale; and finally, cells reach their lifetime limit and undergo senescence (programmed cell aging, whereby a cell line will cease division after a set number of doublings) and death. Adapted from Palsson and Bathia (2004).

The immortal, or continuous cell lines can arise spontaneously (as a rare event) or by transformation either by treatment with carcinogenic chemicals or as a result of exposure to DNA tumor viruses (Doyle and Griffiths, 2000). The viral genes used are suspected to act by blocking the inhibition of cell cycle progression, leading to an increase in life span an enhanced opportunity for mutations and the appearance of an immortalized derivative (Palsson and Bhatia, 2004).

The cells in a cell culture can be classified according to the mode of culture or to the morphology. In the first case cells can be in suspension, not adhered to the surface neither to each other, or in monolayer, adhered to the surface and among them. Concerning the morphology cells can be epithelial (polygonal), fibroblasts (thin and elongated) and others as blood, nerve or muscle cells.

3.1.2 – Cell culture methods

The ability to isolate, manipulate and maintain cells in vitro is a critical phase of cell and tissue culture. This process includes several steps as: cryopreservation, culture maintenance and prevention and detection of external contamination.

Cryopreservation

As it is necessary to keep and maintain cells stored so that they can repeatedly be used from the same "starting" state, the cryopreservation is a determinant step. The cell response to the cryopreservation depends on the method of freezing, especially the cooling rate. If extracellular ice is formed it rises to a chemical potential difference across cell membranes, driving water out of the cell by osmosis. Nevertheless, the plasma membrane has a finite permeability to water, the magnitude of which determines the rate of water efflux and the corresponding time scale of cell dehydration. Thus if the rate of cooling is sufficiently slow to allow the intracellular solution to equilibrate with its external environment by expelling water through the cell membrane, the cell will dehydrate extensively with decreasing temperature. On the other hand, if the cooling rate is fast compared with the rate of water efflux, low temperatures are reached before significant dehydration can occur. In this case, the cell remains largely deformed, but there is a very high probability of ice formation in the cell, as the intracellular solution is in a supercooled nonequilibrium state (Karlsson and Toner, 2000). The effect of cooling rate on the cell survival is presented schematically in Figure 3.3.

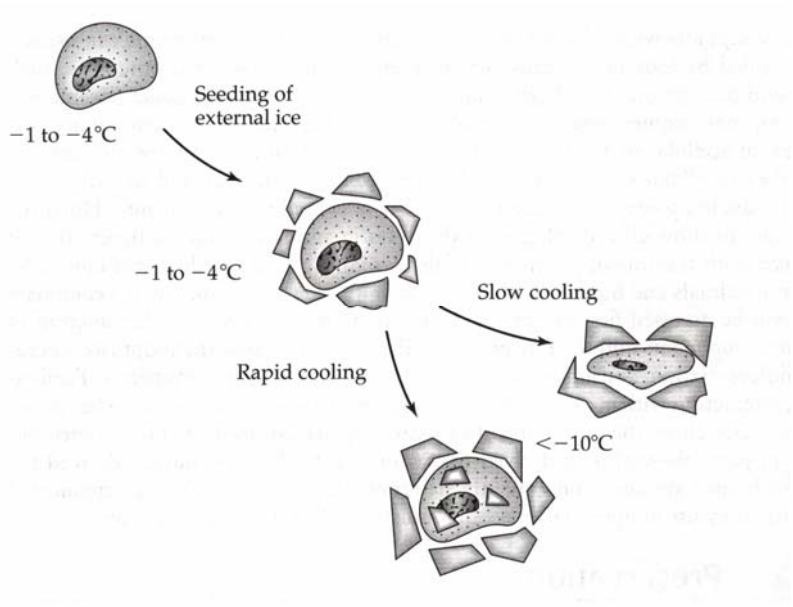


Figure 3.3 – Effect of cooling rate on cell survival. Adapted from Palsson and Bathia (2004).

Thus it can be established an optimum cooling rate at which the two mechanisms of damage are balanced, and the probability of cell survival reaches a maximum (Karlsson and Toner, 2000). Usually, during the freezing process permeant cryoprotective additives such as dimethyl sulfoxide (DMSO) or glycerol are used. The freezing process includes several steps, as keeping the cells at -20 °C, decreasing the temperature to - 80 °C and storing at - 136 °C in liquid nitrogen.

Cell maintenance

The cell maintenance in vitro depends on the liquid in which cells are grown, which provides inorganic salts and other nutrients. The tissue culture media can include:

- basal medium components as, sodium chloride (adjusts osmotic pressure), inorganic salts (provide electrolyte balance similar to blood), sodium bicarbonate (provides buffering capacity), D-glucose (as source of energy, carbon), amino acids (as source of nitrogen for protein synthesis), vitamins (cofactors in various intracellular biochemical reactions) and phenol red (as a visual pH indicator);
- serum, that provides cell growth, attachment factors, hormones and carrier proteins;
- growth factors, hormones that stimulate growth function;
- antibiotics, used to prevent contamination by microorganisms.

The selection of the optimal medium to be used, depends on the culture type, the most common commercially available media are: BME (basal medium Eagle's), EMEM (minimum essential medium with Earl's salts), DMEM (Dulbecco's modified Eagle's medium) and RPMI 1640 (created by Roswell Park Memorial Institute).

The serum used in medium preparation is a fraction of whole blood. Plasma is the noncellular fraction of the blood, whereas serum is the liquid that remains after plasma is allowed to clot. Serum is typically added to culture medium in a proportion of approximately 1 to 20% by volume. Common tissue culture sera are calf (bovine), foetal bovine, horse and human (Palsson and Bhatia, 2004). Despite of the advantages, the use of serum has some disadvantages, namely: the chemically constituents that are note defined or may vary; the extensive testing necessary before use; the lack of reproducibility; the difficulty of standardization of experimental and production protocols; the risk of contamination; the availability and costs; and may contain growth and metabolism inhibitors.

If cells grow adhered to the bottom of a T-flask or a Petri plate it will be necessary to detach them. For that, chemical or physical processes can be used. Among the latter, scrapping is commonly used when the adhesion is not tight. Sometimes chemical detachment is needed and it can be done with digestion enzymes. The enzyme digestion can be performed using different enzymes depending on the tissue type. Examples of enzymes are: collagenase, trypsin, elastase and papain. The most common is trypsin, which is a pancreatic serine protease with specificity for peptide bonds involving the carboxyl group of the basic amino acids arginine and lysine (Doyle and Griffiths, 2000).

In the case of cellular explants it may be necessary to perform some mechanical separation prior to the use of enzymes. This can be achieved by first finely cutting up tissues using sterile scalpels. Ideally pieces of tissue should be 1 mm^3 or smaller if possible. This maximizes the surface area available for enzyme digestion (Papaioannou, 1998).

Contamination

Maintenance of cells in physiological, nutrient-rich environment provides an ideal medium for the growth of "contaminant" microorganisms. Sterile culture techniques and the use of antibiotics are designed to prevent contaminations; however, microbial contaminants are routinely encountered nonetheless. Included in these microbial contaminants are:

- bacteria, fungi and yeasts, that can be detected by an increase in the medium turbidity and a changing in the colour of the medium;
- mycoplasma, that is the most difficult contaminant to detect and on account of that has been of major importance in the last years (Robinson *et al.*, 1956). The detection of mycoplasm includes techniques as staining, culture, DNA probes and co-cultivation (Doyle and Griffiths, 2000).

3.1.3 – Tissue culture methods

The possibility of growing large quantities of differentiated human cells on complex matrices has opened up the possibility of tissue engineering by providing human recipients with replacement transplantable tissues and, in not a too distant future, organs. This technology has developed from advances made in three-dimensional cell culture in which cells are given the correct physical and physiological environment to ensure continued differentiated functions (Doyle and Griffiths, 2000).

One approach to tissue engineering is to create an in vitro environment that embodies the biochemical and mechanical signals that regulate tissue development and maintenance in vivo. According to Freed and Vunjak-Novakovi (1995) the in vitro tissue culture system includes three major components: metabolically active cells able to express their differentiated phenotype; polymeric scaffolds that provide a three-dimensional structure for cell attachment and tissue growth; and bioreactor culture vessels that provide an in vitro environment in which cell-polymer constructs can develop into functional tissues. In Figure 3.4 are presented examples of bioreactor culture vessels.

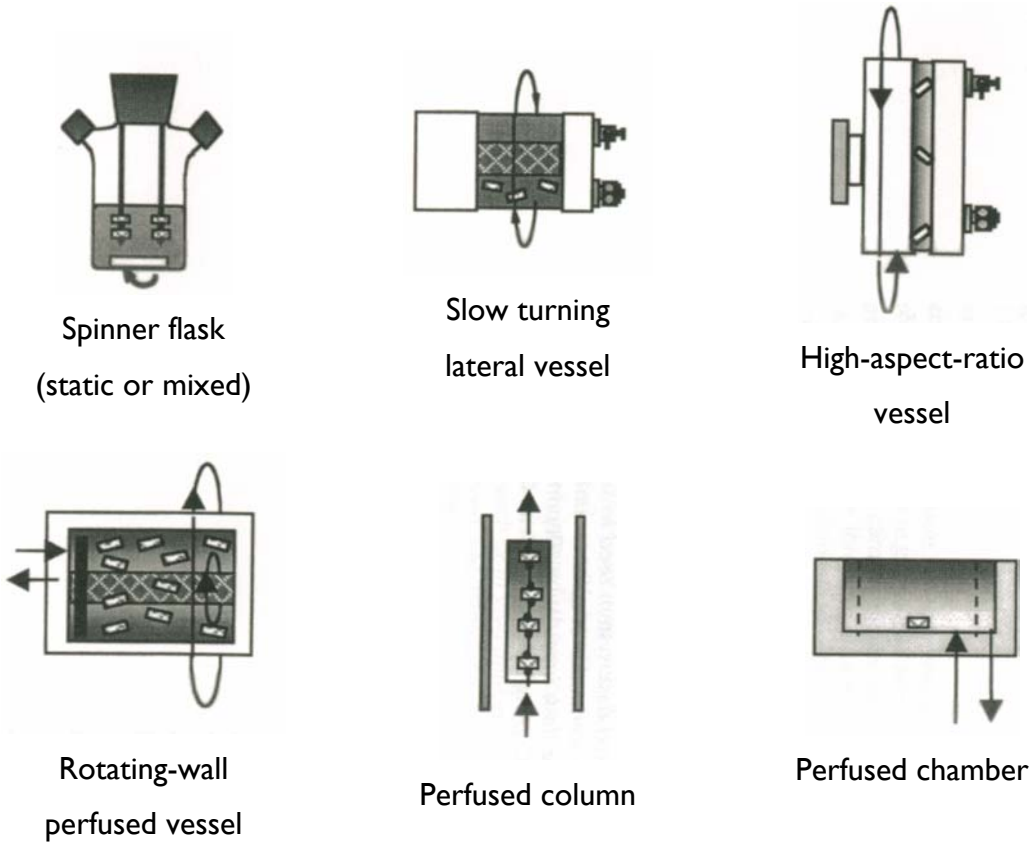


Figure 3.4 – Bioreactor culture vessels. Adapted from Freed and Vunjak-Novakovi (2000).

3.1.4 – Adhesion of *Candida* species to epithelium

The association and subsequent adhesion of microorganisms to host surfaces comprises more than one mechanisms of binding. A scheme of the interaction of a *Candida* cell with an epithelial cell is presented in Figure 3.5

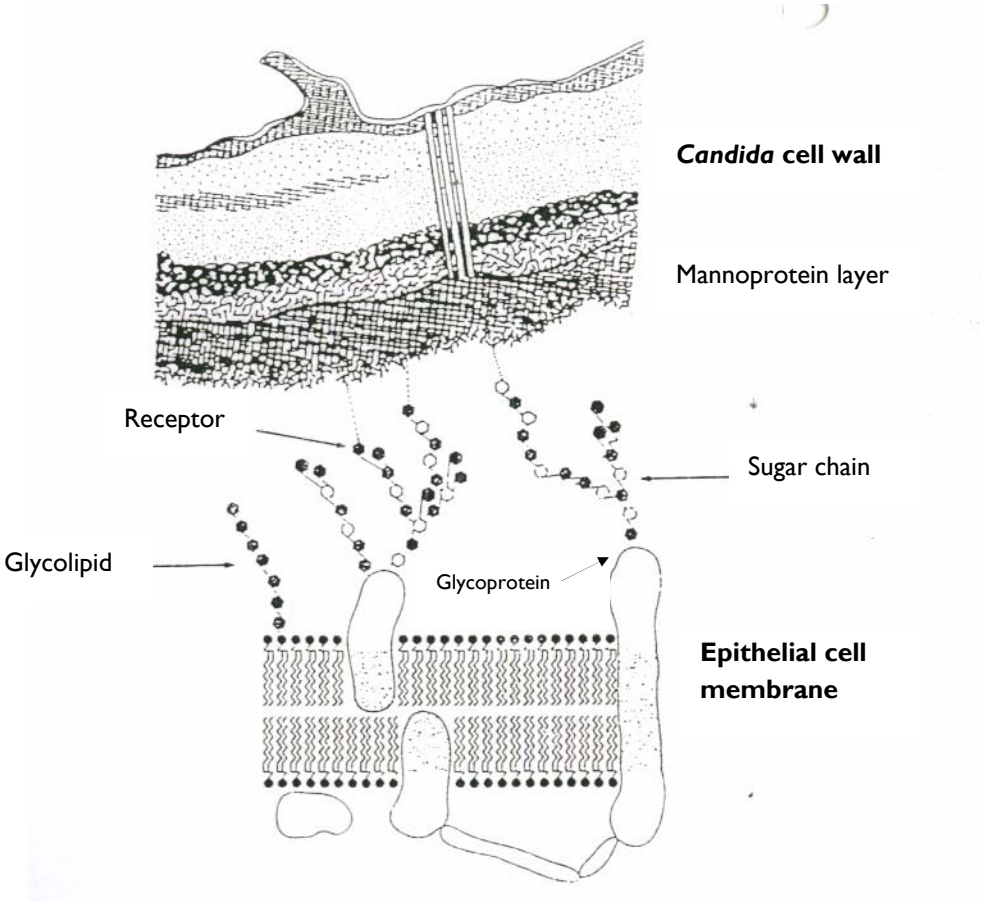


Figure 3.5 – Diagram illustrating the interaction of a *Candida* cell with a mammalian epithelial cell membrane. Adapted from Ghannoum and Radwan (1990).

As it presented in Figure 3.5 the outer surface of the yeast cell wall is a mass of mannoproteins fibrils attached to the polysaccharides extending from the epithelial membrane.

The interactions, established between yeast cells and human tissue, depend on the yeast cell characteristics, on the liquid where adhesion takes places and on the type of interaction.

Yeast cells

There are several characteristics of *Candida* species that can influence adhesion to epithelial cells, namely:

- Species and strain: *C. albicans* is undoubtedly the most virulent *Candida* species, followed by *C. tropicalis* (Ghannoum and Abu-Elteen, 1991). The difference in the virulence is closely paralleled by differences in the ability of the organisms

to adhere to epithelial cells. Differences in adhesion between strains of the same species are less obvious, but were reported in some studies (Douglas, 1987);

- Culture and age viability: growth phase of *C. albicans* has a marked influence on its adherence ability;
- Culture medium and temperature of growth: medium composition and temperature of growth are known to affect cell morphology of dimorphic fungi including *C. albicans* (Ghannoum and Abu-Elteen, 1991), thus affecting yeast cell-surface composition. Optimal adhesive activity was observed when the cells were grown in defined medium (Kennedy and Sandin, 1988);
- Germ-tube formation: It was demonstrated that the presence of saliva in assay mixtures incubated at 37 °C resulted in increased adherence to buccal epithelial cells (BEC), which appeared to be associated with germ-tube formation (Kimura and Pearsall, 1978);
- Culture concentration: there is no detectable yeast attachment at concentrations bellow 10^4 cells ml^{-1}. It has been reported that attachment of *C. albicans* to BEC/VEC (vaginal epithelial cells) gradually increases as the ratio of yeasts to epithelial cells in incubation mixtures is raised from 10:1 to 10000:1 (Kimura and Pearsall, 1978; Lee *et al.*, 1997).

Adhesion media

The adhesion phenomenon of *Candida* species occurs in the presence of body fluids that have special characteristics which may influence adhesion. Adhesion of *C. albicans* to VEC is affected by the pH and CO_2 levels present in the adhesion medium. The greatest adhesion ability was observed (Persi *et al.*, 1985) when the yeast cells were incubated with VEC at pH 5 in PBS in ambient air supplemented with 10% CO_2. The presence of antibodies can also influence *Candida* adhesion. Kimura and Pearsall (1978) showed that antibodies against *C. albicans* inhibit adherence to BEC in vitro.

Adhesion interactions

The interactions involving yeast cells and epithelial cells can be non-specific or specific. The non-specific interactions can include hydrophobic and electrostatic forces. These interactions were already described in Chapter 2.

Concerning the epithelial surfaces, the specific interactions involved in adhesion are:

- Endothelial cells/ endothelial extracellular matrix adhesion molecules: a number of proteins located within the endothelial extracellular matrix (ECM) have been implicated as possible targets for *C. albicans* adherence. These include fibronectins, collagen types I and IV and laminin. Molecules present on the surface of *C. albicans* are thought to mediate adherence to ECM molecules, and are classified as integrin analogues because of their structure and functional homology to mammalian integrins.
- Endothelial C3d and iC3b adherence molecules: receptors for C3 degradation fragments (e.g. iC3b) are presented on many cells of the host immune system, and also can be found on a number of microorganisms which are pathogenic to humans. iC3b receptors are present on the surface of *C. albicans*, and these share homology with a subunit of the neutrophilic iC3b receptor (Lee K.H. *et al.*, 1997), and it is proposed that a number of fungal proteins mediate *C. albicans* adherence to iC3b receptors (CR3-like).
- Epithelial-cell receptor-ligand interactions: adherence of *C. albicans* to epithelial cells appears to be achieved by a simpler mechanism than those involving endothelial cells. Most experimental evidence supports the role of mannoproteins in mediating the adherence of *Candida albicans* to epithelial cells (Cotter and Kavanagh, 2000). Several authors have observed that the adherence of *C. albicans* blastopores to buccal epithelium involves binding to glycoside receptors on the host-cell surface.
- Miscellaneous adherence mechanisms: it was proposed that a developmentally regulated gene (HWP1), expressed in germ-tube and hyphal forms of *C. albicans*, encodes an outer cell-wall mannoprotein that interacts with epithelial cell transglutaminase, forming a non-dissociable complex (Staib *et al.*, 1999).

3.1.5 – Methods to study the adhesion of *Candida* species to epithelium

The attachment or adherence of a microbe to a tissue or mammalian cells precedes all infectious diseases. There are, however, exceptions as for example the case of adhesion of lactobacillus species or other probiotics to intestinal mucosa.
To study adhesion of *Candida* to epithelium three major steps are involved, the preparation of the epithelial cells, the adhesion assay and the quantification of adhesion extent.

Epithelial cells

The exfoliated epithelial cells can be easily obtained from human volunteers by gently swabbing or scraping the mucosal surface (Douglas, 1987). Because of their ease to grow, monolayers are most commonly used. However, such cultures mimic neither the differentiation of cells during maturation, nor their interactions encountered *in situ*. A more realistic model seems to be reached with the combination of stromal equivalent and epithelial cells, commonly called organotypic cultures (Papaioannou, 1998).
Although this type of cells is convenient to use, invariably consists of heterogeneous mixtures of viable and non-viable cells and substantial cell-to-cell variation in the number of adhered yeast is always observed. Such preparations may also vary according to the donor, the time of sampling, the extent of colonization by the normal flora and the degree of exposure to various secretions. So, it is more convenient to use a uniform cell population obtained by culturing epithelial cells. The most common cultured cells are HeLa, CCL-6 endothelial cells and fibroblasts. HeLa cells have origin in a human carcinoma of the cervix and are the most studied cell line. Among the cultured exfoliated cells are BEC, VEC, uroepithelial and corneocytes epithelial cells.

Adhesion assays

The attachment of *Candida albicans* to buccal cells from rats was first measured by Liljemark and Gibbons (1973). These first studies included the preparation and mixing of equal volumes of standardized suspensions of yeast and epithelial cells. Although this method is still being used (Nair and Samaranayke, 1996b; Pizzo *et al.*, 2001), in 1982

Samaranayke and MacFarlane (1982) described a new method of studying adhesion to epithelium that includes the adhesion of epithelial cells to the bottom of a well plate or to glass coupons (inserted on the wells) prior to adhesion of microbial cells. In this method mammalian cells must be adherent to the supporting surface.

Adhesion quantification

Among the methods used to enumerate yeast cells adhered to epithelium are: the visual method, using light, fluorescence and electron or scanning transmission microscopy and the Coulter count of the radiolabelled yeast. The visual method allows monitoring of adhesion to individual epithelial cells but it is a very time consuming technique. The radiolabelling method seems to offer an attractive alternative in some situations, although it should always be remembered that leachable isotopes can produce misleading results.

3.1.6 – Aims

Adherence of fungi to mammalian cells has not been studied to the same extent as bacterial adherence. In fact, most of the experiments on fungal adherence to host cells have been done mostly with *Candida albicans*. Although there are several studies including adherence of *Candida albicans* to epithelium (Bailey *et al.*, 1995; Dorocka-Bobkowska *et al.*, 2003; Hoffman and Haidaris, 1993; Pendrak and Klotz, 1995; Phan *et al.*, 2000; Segal and Sandovsky-Losica, 1995), concerning *Candida dubliniensis* there are only few studies (Jabra-Rizk *et al.*, 2001b; Vidotto *et al.*, 2003). So, one of the main goals of the part of the work described in the present chapter was the study of both *Candida albicans* and *Candida dubliniensis* to epithelial cells. In order to mimic the oral conditions, an artificial saliva solution, besides the saline solution, was used as adhesion medium.

3.2 – Materials and Methods

3.2.1 – Media

Cells were grown in Sabouraud dextrose broth (SDB - Merck), that is the most common medium used to grow *Candida* species or in an artificial saliva medium. The adhesion assays were performed either in ultrapure water, artificial saliva solution or saline solution.

Sabouraud dextrose medium

The yeast cells were maintained in Sabouraud dextrose agar (SDA) that was prepared according to the manufacturer's instructions (30 g l^{-1}) and 1.7 % of agar (Merck) was added. Sabouraud dextrose broth (SDB – Merck) that was prepared using 30 g l^{-1} in water, was used when the cells were grown in liquid medium.

EMEM medium

The medium used to grow epithelial cells was prepared by adding 10 % of foetal bovine serum (Sigma-Aldrich), 2 mM of Glutamine (Sigma-Aldrich) and 1 % Non Essential Amino Acids (Sigma-Aldrich) to EMEM medium (Sigma-Aldrich).

Artificial saliva

In some experiments artificial saliva was used to mimic the *in vivo* oral conditions. This saliva was prepared according to Gal *et al.* (2001) with the following composition in mg l^{-1}: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5 KH_2PO_4, 200.0 Urea, 763.2 $Na_2SO_4.10H_2O$, 178.0 NH_4Cl, 227.8 $CaCl_2.2H_2O$ and 630.8 $NaHCO_3$. The pH was adjusted with carbon dioxide to 6.8.

Saline solution

To avoid epithelial cells disruption by osmotic shock, a saline solution (SS) 0.9 % (w/v) of NaCl in water was used.

3.2.2 – Yeast cells

The *Candida* species studied were *Candida albicans* and *Candida dubliniensis* and two different strains of each species were used. In the case of *Candida albicans* one strain from the American Type Culture Collection, ATCC 32354 (*Candida albicans* B311) and a clinical isolate (*Candida albicans* 12A) were used. *Candida dubliniensis* cells were obtained from Centraalbureau voor Schimmelcultures (CBS) (*Candida dubliniensis* 7987 and *Candida dubliniensis* 7988).

For all the assays the yeast cells were grown for 24 h in SDA at 37 °C. The cells were then inoculated in SDB for 18 h at 37 °C and 150 rpm. After incubation cells were harvested by centrifugation for 10 min at 5000 rpm and 4 °C.

3.2.3 – Epithelial cells

The HeLa cells were kindly provided by Drª Elsa Anes from the Faculty of Pharmacy, University of Lisbon.

After being slowly defrosted cells were added to a falcon tube containing 9 ml of medium and centrifuged for 5 min at 800 rpm. The pellet was resuspended in 2 ml of medium and then the suspension was added to a T-flask containing 3 ml of fresh medium. The flask was maintained in a CO_2 incubator at 37 °C until 80 % of confluence was obtained. At this point, the medium was removed and the cells were washed once with 2 ml of saline solution. After discarding the SS, 1 ml of trypsin was added and the cells were kept for 10 min at 37 °C until they detached from the flask. To stop the trypsin activity 3 ml of medium were added to the flask. Cells were enumerated in a Neubauer chamber and were then diluted in 5 ml of medium to 1×10^6 cells ml^{-1}. The new flasks were incubated at 37 °C with 5 % of CO_2. The trypsinization was repeated to prepare new flasks – to maintain the cells, or to prepare cells for adhesion assays. In the latter, after detachment, 10^5 cells ml^{-1} were added to a 24 well

plate containing circular glass lamellas (ϕ = 12 mm) in the bottom. After reaching confluence the cells were washed two times with SS and were ready to be used in the adhesion assays.

3.2.4 – Adhesion assay

To perform the adhesion assays, 2 ml of the microbial cell suspension (10^7 cells ml^{-1} prepared with water or saliva) were added to each well where a coupon of substratum was previously inserted. After 1 h of incubation (100 rpm, at 37 °C) each well was washed twice with SS, by removing carefully only the liquid above the coupon. Finally, all the liquid was removed.

The period of incubation was determined based on the kinetics of cell adhesion present in Figure 3.6.

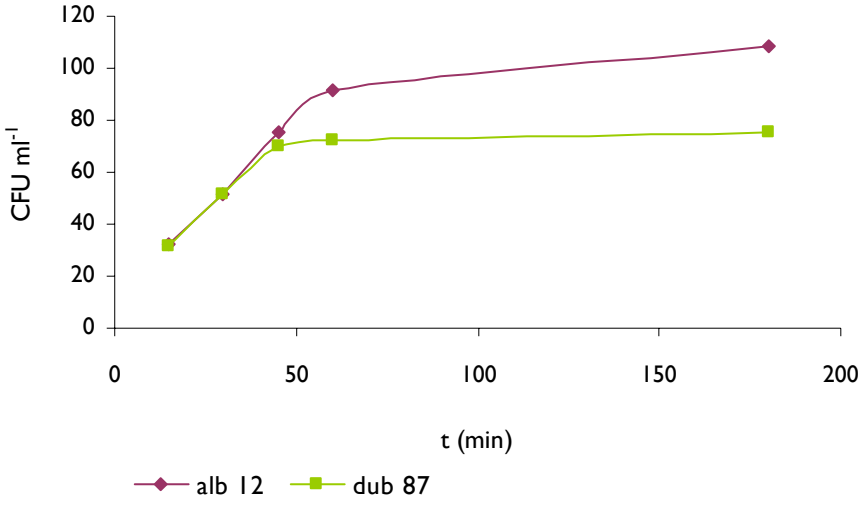


Figure 3.6 – Kinetics of *Candida albicans* 12A (alb 12) and *Candida dubliniensis* 7987 (dub 87) adhesion to epithelial cells.

As in the case of adhesion to inert materials (Chapter 2), the number of yeast cells adhered to epithelial cells achieved a plateau after 60 min of contact with the surface.

Cell enumeration

The glass coupons were withdrawn from the wells and were Gram stained to be observed under contrast microscopy. The images were captured with a video camera that was coupled to the microscope and connected to a computer. Twenty five fields were randomly counted in each sample. Each experiment was repeated three times. In each image, all epithelial cells were enumerated distinguishing the isolate epithelial cells and the epithelial cells with one or more attached yeast cells.

3.2.5 – Contact angle measurement

Cell lawns were prepared by vacuum filtering a cell suspension of 10^9cells ml^{-1}, through a 3 µm membranes. The membrane was cut in three parts and placed in a Petri dish containing 20 g l^{-1} of agar, and 10% (v/v) of glycerol for 2h30min. Contact angles were measured by the sessile drop technique using an apparatus model OCA 15 PLUS, DATAPHYSICS.

The measurements were performed at room temperature, using three different liquids: water, formamide and α-bromonaphthalene. Each assay was performed in triplicate and at least 10 contact angles, per sample, were measured.

The free energy and surface tension parameters calculations are presented in Chapter 2.

3.2.6 – Statistical analysis

The data were statistically analysed using SPSS (Statistical Package for the Social Sciences). One way ANOVA with Bonferroni test was used for the different comparisons. All tests were performed with a confidence level of 95%.

3.3 – Results

The average number of yeasts adhered to one epithelial cell in saline medium and in artificial saliva is presented in Figure 3.7.

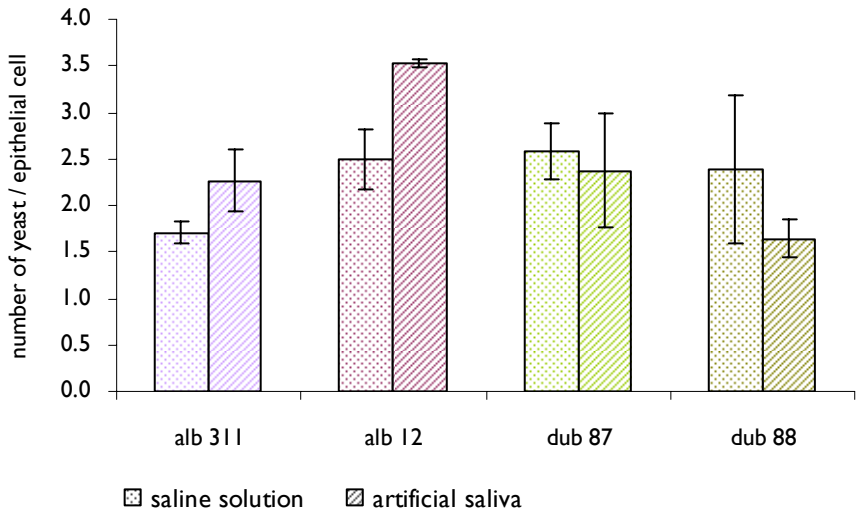
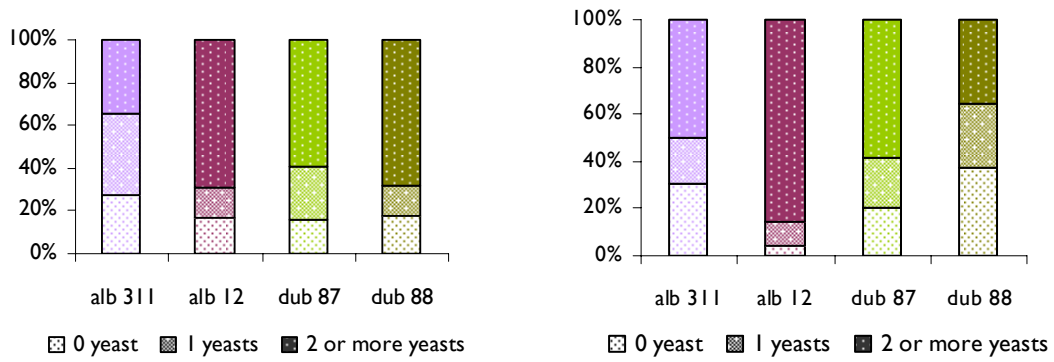


Figure 3.7 – Average number of *Candida albicans* B311 (alb B311), *Candida albicans* 12A (alb12), *Candida dubliniensis* 7987 (dub87) and *Candida dubliniensis* 7988 (dub88) adhered to one epithelial cell in saline solution and in artificial saliva.

There was no significative difference in the number of adhered *Candida albicans* 12A and both strains of *Candida dubliniensis* in the presence of saline solution ($p>0.05$). In saliva, while the number of adhered *Candida albicans* 12 A and *Candida dubliniensis* 7988 presented significant differences ($p<0.05$), the number of adhered *Candida albicans* B311 and *Candida dubliniensis* 7987 were not statistically different ($p>0.05$). In saline solution, *Candida albicans* B311 presented the lower number of adhered cells. However, in the presence of artificial saliva the lower number of yeasts per epithelial cell was obtained for *Candida dubliniensis*. Comparing the adherence behaviour in both media all the strains showed significative differences ($p<0.05$). It is interesting to note that the number of both strains of *Candida albicans* adhered to epithelium increased in the presence of artificial saliva, while the number of adhered *Candida dubliniensis* decreased.

The observation of the microscope images made possible to calculate the percentage of HeLa cells without adhered *Candida*, with one and with two or more yeasts per cell.



(a) (b)

Figure 3.8 – Percentage of epithelial cells without yeasts adhered, with one yeast or with two or more in the presence of saline solution (a) or artificial saliva (b).

In the case of *Candida albicans* 12A there was a decrease in the percentage of HeLa cells without yeasts and an increase in the percentage of HeLa cells with two or more yeasts per cell, from saline solution to artificial saliva (Figure 3.8). On the opposite, in artificial saliva, the number of HeLa cells without *Candida dubliniensis* 7987 or *Candida dubliniensis* 7988 increased while the number of HeLa cells with two or more yeasts increased, these changes were more significant for the second strain. Comparing the number of *Candida albicans* B311 adhered in the presence of saline solution or in artificial saliva, there was no alteration in the percentage of epithelial cells without yeasts; the amount of HeLa cells with one yeast decreased and the number of HeLa cells with two or more yeasts increased.

The adhesion phenomenon can be closely related to the surface physico-chemical properties of microbial cells. The relevant surface properties, likewise the surface tension parameters and the degree of hydrophobicity of the four strains are presented in Table 3.2. These properties were determined based on the values of the contact angles of water, formamide and α-bromonaphtalene (Table 3.1).

Table 3.1 – Values of contact angles measured with water (θ_w), formamide (θ_f) and α-bromonaphtalene (θ_b) on cells lawns of *C. albicans* 12A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 conditioned with water or artificial saliva

Medium	Cells	θ_w (°) (±SD[a])	θ_f (°) (±SD[a])	θ_b (°) (±SD[a])
Saline solution	*C. albicans* B311	19 ± 1	12 ± 1	49 ± 1
	C. albicans 12A	16 ± 2	19 ± 1	47 ± 0
	C. dubliniensis 7987	16 ± 0	22 ± 3	55 ± 2
	C. dubliniensis 7988	13 ± 1	16 ± 2	44 ± 1
Artificial Saliva	*C. albicans* B311	15 ± 2	16 ± 1	63 ± 7
	C. albicans 12A	13 ± 1	16 ± 3	83 ± 1
	C. dubliniensis 7987	13 ± 2	14 ± 2	62 ± 5
	C. dubliniensis 7988	10 ± 1	12 ± 2	61 ± 5

[a]SD, Standard Deviation

Table 3.2 – Values of the surface tension parameters (γ^+,γ^-,γ^{LW}) and degree of hydrophobicity (ΔG_{sws}) of *Candida albicans* B311 (alb B311), *Candida albicans* 12A (alb12), *Candida dubliniensis* 7987 (dub87) and *Candida dubliniensis* 7988 (dub 88) determined in saline solution and in artificial saliva

Medium	Cells	γ^+ (mJ m^{-2}) (±SD[a])	γ^- (mJ m^{-2}) (±SD[a])	γ^{LW} (mJ m^{-2}) (±SD[a])	ΔG_{sws} (mJ m^{-2}) (±SD[a])
Saline Solution	*C. albicans* B311	3 ± 1	46 ± 2	29 ± 1	21 ± 1
	C. albicans 12A	4 ± 1	51 ± 3	27 ± 5	25 ± 5
	C. dubliniensis 7987	3 ± 0	52 ± 1	30 ± 0	27 ± 1
	C. dubliniensis 7988	4 ± 2	40 ± 11	39 ± 9	24 ± 6
Artificial Saliva	*C. albicans* B311	6 ± 1	51 ± 1	23 ± 1	21 ± 4
	C. albicans 12A	3 ± 0	49 ± 3	32 ± 1	24 ± 2
	C. dubliniensis 7987	6 ± 1	52 ± 0	24 ± 2	22 ± 2
	C. dubliniensis 7988	6 ± 2	53 ± 1	25 ± 3	23 ± 4

[a]SD, Standard Deviation

The value of the free energy between two identical entities immersed in an aqueous medium (ΔG_{sws}) represents the degree of hydrophobicity of that entity. If $\Delta G_{sws}>0$ the surface under concern can be considered as having a hydrophilic character and on the opposite, if $\Delta G_{sws}<0$, the surface is hydrophobic. In the present case (Table 3.2) all the yeast strains either in saline solution or in artificial saliva, present a hydrophilic character, which confirms the fact that the water contact angle values (Table 3.1) are all lower than 50° (van Oss and Giese, 1995).

Hyphae formation is also an important factor that can be determinant in the adhesion phenomenon.

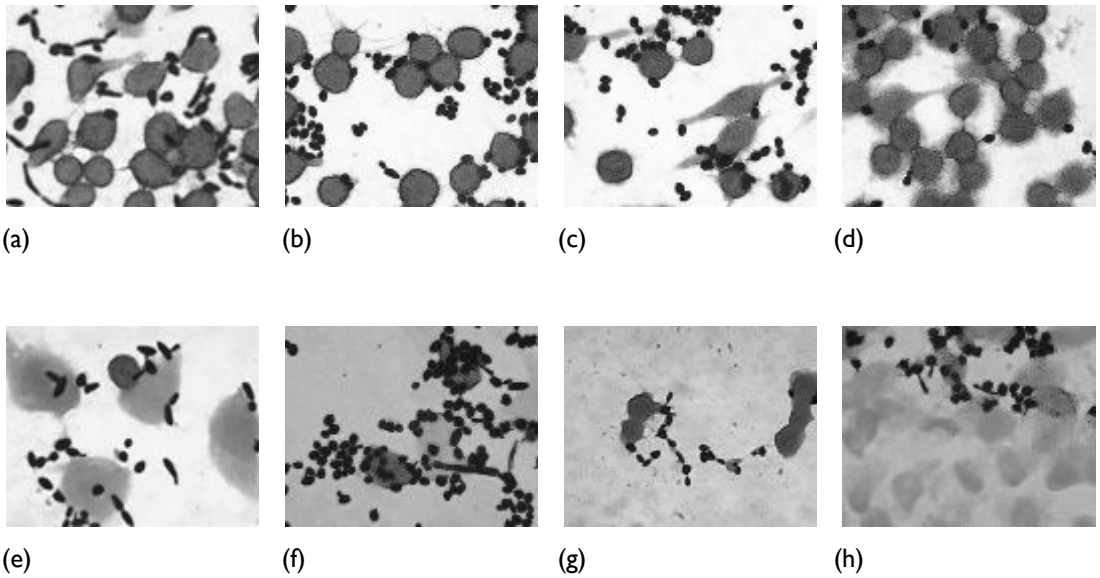
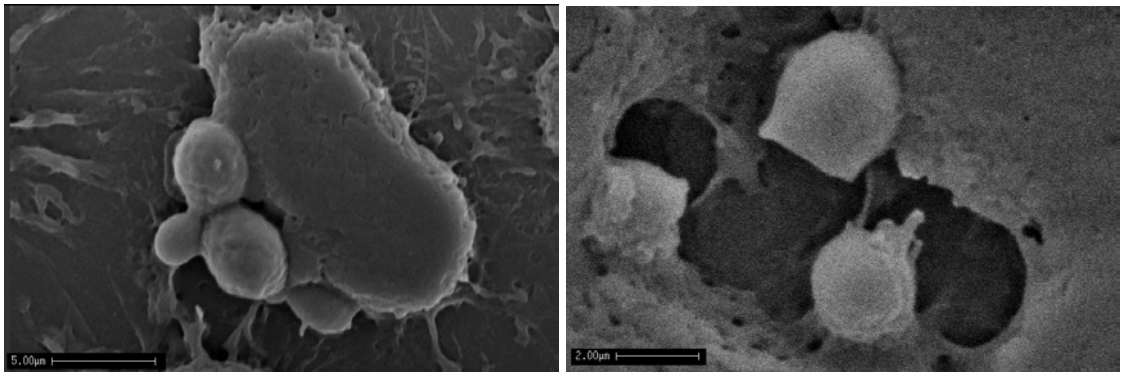


Figure 3.9 – Images of *Candida albicans* B311 (a and e), of *Candida albicans* 12A (b and f), *Candida dubliniensis* 7987 (c and g) and *Candida dubliniensis* 7988 (d and h) adhered to epithelium in saline solution (a, b, c and d) or artificial saliva (e, f, g and h).

From Figure 3.9 it is possible to see that *Candida albicans* B311 formed pseudohyphae either in saline solution or in artificial saliva. The other strains formed hyphae only in the presence of artificial saliva.

The microscopic observations revealed that yeast cells adhered preferentially to the borders of the outer surface of epithelial cells. Scanning electron microscopy (SEM) images of samples from the adhesion assays of *Candida albicans* 12A also corroborated this observation (Figure 3.10).



(a) (b)

Figure 3.10 – Images of *Candida albicans* 12A adhered to epithelial cells observed by SEM with 4500x (a) and 9000x (b) of magnification. The bar represents 5.0 µm in the figure (a) and 2.0 µm in the figure (b).

3.4 – Discussion

The most common in vitro models of mammalian cell lines used to study *Candida* infection, are exfoliated BEC´s (Buccal Epithelial Cells), vaginal, urogenital and corneal cells. However, problems arise with exfoliated epithelial cell preparation, due to the presence of heterogeneous populations that show an abundance of non-viable cells, bacterial contamination and different degrees of enzymatic modifications of the cell surface. To avoid such problems, the use of uniform populations of cells in measuring adherence has been developed. These include HeLa and human embryonic kidney epithelial cells, fibroblasts and HEp-2 cells (Cotter and Kavanagh, 2000). A HeLa cell line was used in this study and all *Candida* strains presented high levels of adherence to this cellular line. The extent of yeast cell adhesion in saline solution, evaluated by the number of yeasts attached to each HeLa cell was very similar for all strains. Conversely, when the adhesion medium was artificial saliva, *Candida albicans* 12A adhered in a significant larger extent than *Candida dubliniensis* 7988, although *Candida albicans* B311 and *Candida dubliniensis* 7987 presented no differences. Vidotto *et al.* (2003) studied the adhesion of *Candida albicans* and *dubliniensis* to buccal and vaginal epithelial cells in the presence of phosphate saline buffer and they found differences in the adhesion behaviours, being *Candida albicans* the strain that presented a higher extent of adhesion. However, Gilfilland *et al.* (1998) showed that oral *C. dubliniensis*

isolates were more adherent to BEC than *C. albicans* when grown in glucose and equally adherent when grown in galactose. Nevertheless, according to several authors (Calderone *et al.*, 2000; Gale *et al.*, 1998; Pereiro *et al.*, 1997), the greater extent of adhesion of some strains of *Candida albicans* with respect to *C. dubliniensis* to buccal and vaginal epithelial cells is in agreement with the fact that *C. albicans* is usually considered more virulent than *C. dubliniensis* (Vidotto *et al.*, 2003).

The adhesion of these strains to acrylic and HAP was also studied (Henriques *et al.*, 2004) and no differences among the strains were encountered, either in water or in saliva as well. This similar behaviour was explained by the similar surface properties (zeta potential, surface tension and hydrophilicity) of the four strains. It is well documented that the adhesion phenomenon to inert surfaces is ruled by physico-chemical properties of microbial cell surfaces. It is not clear, however, if these properties also determine microbial adhesion to human epithelium.

No relation was found between yeast cell surface tension or hydrophobicity (Table 3.2) and the ability to adhere to epithelium. So, as in the case of adhesion to inert surfaces, hydrophobic properties do not role adhesion. However, while the electron donor parameters were found to be responsible for the adhesion phenomenon to inert surfaces, in the case of adhesion to epithelium this was not evident.

It must be stressed that the extent of yeast adhesion to epithelial cells can only be compared with that to inert surfaces in a qualitative way, since in the first case it was the number of yeasts per epithelial cells that was determined, while on the inert surfaces it was the number of yeasts per mm^2 that was quantified. There are some authors (Dorocka-Bobkowska *et al.*, 2003) that have measured the number of yeasts per mm^2 in the case of adhesion to epithelial cells, although this is only possible if the cells are 100% confluent which is difficult to achieve.

In the present study, the number of cells of both strains of *Candida albicans* adhered to HeLa cells increased in the presence of artificial saliva, while the contrary happened for *Candida dubliniensis* strains.

The role of saliva in the adhesion to epithelium has been largely studied in the case of *Candida albicans*. Although some authors (Holmes *et al.*, 2002) found that saliva promotes the adhesion of *Candida albicans* to epithelial cells, others (Umazume *et al.*,

1995) report the opposite. So, the influence of saliva in adhesion depends on various factors, such as the origin and composition and also on the strain of *Candida* that is being studied.

The factors affecting *Candida* adhesion to epithelial cells can depend on the yeasts, on the epithelial cells or on environmental factors. Within yeast factors can be included cell concentration and viability, the growth phase and temperature, the growth-medium composition, species and strains and germ-tube formation (Douglas, 1987).

Candida albicans and *Candida dubliniensis* are the two *Candida* strains with capacity to form true hyphae in addition to pseudohyphae (Calderone, 2002c). The transition from yeast to hyphae form is one factor of *Candida* virulence. Hyphae formation depends on the medium used to grow the yeast cells and the number of formed hyphae increases with time (Gilfillan *et al.*, 1998). In this study *Candida albicans* B311 presented hyphal formation in both adhesion media, saline solution and artificial saliva. While the other strains formed hyphae only in the presence of artificial saliva.

Although the cells were grown in SDB and put in contact with artificial saliva only during the adhesion assay (one hour), all the strains studied presented hyphal formation in this case. Other authors (Gilfillan *et al.*, 1998) also found that hyphae formation can occur after one hour either for *Candida albicans* or *Candida dubliniensis* in different media.

The environmental factors that favour germination, formation of pseudohyphae or hyphae, include a temperature greater than 35 °C and a pH of 6.5 to 7 or slightly alkaline (Calderone, 2002c). The pH of the artificial saliva used is 6.8 what can explain the formation of hyphae by all the strains.

According to Nikawa *et al.* (2002), in some *Candida albicans* strains the adhesion capability increases in the presence of germ tubes when comparing to blastopores. However, for other strains no significant differences were noticed.

Hyphal formation did not seem to play an important role in the adhesion of *Candida*. So, other factors, rather than physico-chemical properties or hyphal formation are ruling the process of adhesion. Among these factors are the peripherical proteins that promote adhesion, called adhesins. A number of proteins have been identified that recognize host cell ligands, including MP60, MP58, MP66, MP130 and MP37 (Sturtevant and Calderone, 1997).

Ultrastructural evidence indicates that specific interactions between *Candida* and epithelial cells are mediated by a flocular-fibrillar adhesin layer present on the outer surface of the yeast (Ghannoum and Radwan, 1990). The *Candida* surface is enriched with concavalin A binding sites and attachment to the epithelial cells is mediated by fibrillar structures or polysaccharide granules distributed on the cell wall coat (Ghannoum and Radwan, 1990).

Candida albicans and *Candida dubliniensis* have equal abilities to adhere to oral surfaces (Henriques *et al.*, 2004) and the adhesion is enhanced in the presence of artificial saliva due to an increase in the physico-chemical interactions. Considering adhesion to epithelium other factors, rather than physico-chemical seem to rule the phenomenon. Additionally clearly differences in the adhesion capabilities were shown among the strains. So, adhesion to epithelium is strain dependent, conversely to adhesion to inert surfaces.

Chapter 4 – BIOFILMS EVALUATION

4.1 – Introduction

The biofilm definition is not unanimous among all the biofilm researchers. One definition based on the morphological structure and generally accepted by the scientific community considers biofilms as: "Complex communities of microorganisms attached to a surface or interface enclosed in a exopolysaccharide matrix of microbial and host origin to produce a spatially organized three-dimensional structure" (Costerton *et al.*, 1999).

The first example of a biofilm to be recognized in medical systems was dental plaque on tooth surfaces, but recent estimates suggest that a substantial proportion of human infections involve biofilms (Potera, 1999). Many of these are implant-related infections in which adherent microbial populations are found on the surfaces of indwelling devices such as catheters, prosthetic heart valves and joint replacements (Donlan, 2001). Biofilm infections can be caused by a single microbial species or by a mixture of bacterial or fungal species (Costerton *et al.*, 1999; Jenkinson and Douglas, 2002). Biofilms are notoriously difficult to eliminate and are a source of many recalcitrant infections (Donlan, 2002; Lewis, 2001). The biofilm formation has been largely studied concerning the use of antifungal agents (Bachmann *et al.*, 2002; Baillie and Douglas, 2000; Kuhn *et al.*, 2002a).

4.1.1 – Biofilm structure

The overall organisation of a Candidal biofilm is generally similar to that of a bacterial biofilm but the details of a *Candida* biofilm structure are highly dependent upon the conditions under which the biofilm is formed. This plasticity in structure suggests that biofilms formed in the human host may also vary depending upon the nature of the implanted device and in location (Kumamoto, 2002).

There is a wide diversity of biofilm structures and architectures. Regardless of the diversity, biofilm structure has a number of common features that have been used for identification. These features include: a substratum to which the microorganisms are

attached, a conditioning film, the biofilm matrix and the liquid or gas phase in contact (Figure 4.1).

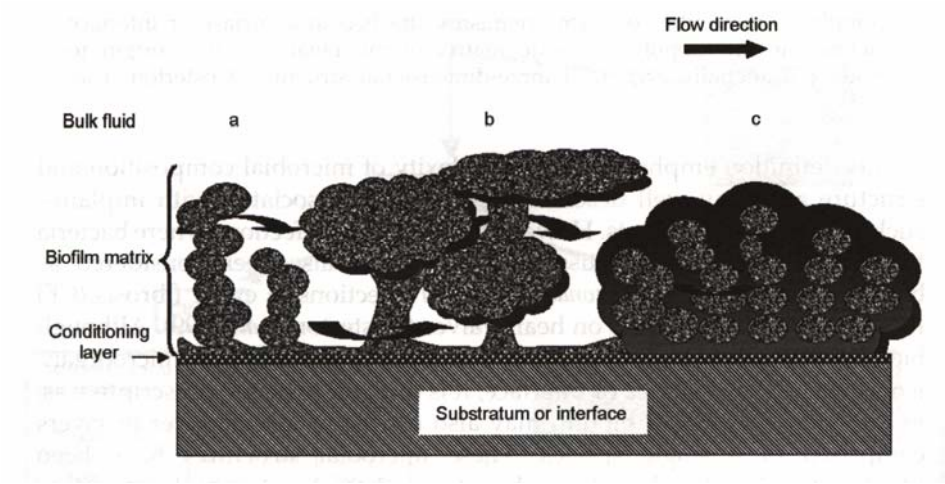
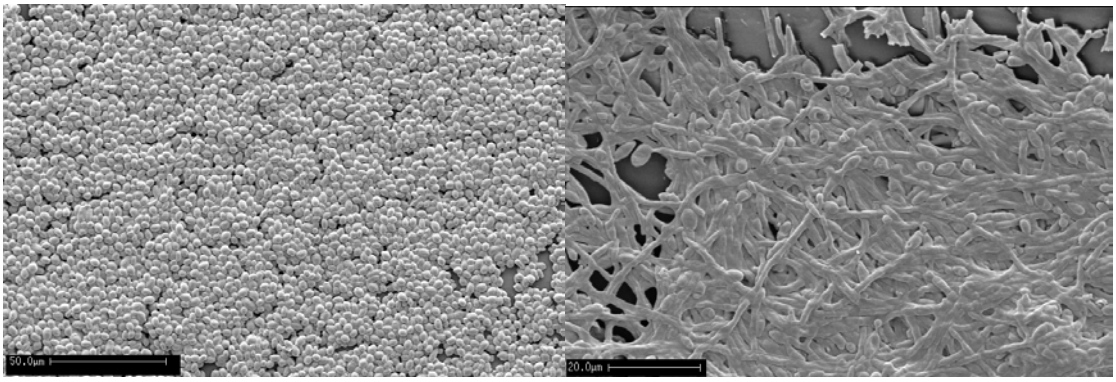


Figure 4.1 – Schematic representation of biofilm structures: heterogeneous mosaic (a), mushroom-like structures (b) and dense-confluent biofilm (c). Adapted from Jass *et al.* (2003).

The heterogeneous mosaic (Figure 4.1a) is characterized by a basal layer and stacks of microcolonies extending up into the aqueous phase. The porous biofilm (Figure 4.1b) is illustrated with mushroom-like structures interdispersed with water channels. The dense-confluent biofilm (Figure 4.1c) appears more tightly packed, often containing multiple species of bacteria with regions of lower density that may act as transport channels within the biofilm (Jass *et al.*, 2003).

The detailed structure of a mature Candidal biofilm (at least 48h old) has been shown to consist of a dense network of yeasts, hyphae and pseudohyphae (Figure 4.2).



(a) (b)

Figure 4.2 – SEM observation of a general view of a mature biofilm – 500 X (a) and a magnification - 1000X of the biofilm containing blastopores, hyphae and pseudohyphae. The bar corresponds to 50 μm in the case of image (a) and to 20 μm in the case of image (b).

This mixture of yeasts, hyphae and matrix material is not seen when the organism is grown in liquid culture or on agar surface, which suggests that morphogenesis is triggered when an organism contacts a surface and that the basal cell layer may have an important role in anchoring the biofilm to the surface (Chandra *et al.*, 2001; Douglas, 2003; Douglas, 2002; Ramage *et al.*, 2001).

Several parameters, surface material (Hawser and Douglas, 1994), medium (Jin *et al.*, 2004), the presence of other microorganisms (Adam *et al.*, 2002) and conditions of incubation (Chandra *et al.*, 2001) can influence the structure of the resulting biofilm and the morphology of cells within it. The demonstration that different conditions lead to different morphologies suggests that both morphological forms, yeast cells and hyphal cells, are capable of biofilm formation. To confirm this, Baillie and Douglas (1999) observed that a morphological mutant unable to form hyphae formed a dense biofilm composed of yeast cells, whereas a mutant that produced only filamentous cells formed a hyphal biofilm.

The role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood. Indeed, components of saliva or serum proteins, such as mucins, fibrinogen and complementary factors specifically bind to *Candida* blastopores and germ tubes (Bouali *et al.*, 1986; Tronchin *et al.*, 1987), possibly modifying the biofilm formation (Nikawa *et al.*, 2000).

Although most implant infections are caused by a single pathogen, polymicrobial infections involving catheters and orthopaedic prostheses have been reported (Adam *et al.*, 2002; Costerton *et al.*, 1985). Mixed bacterial-fungal biofilms are also associated

with infections of endotracheal tubes, biliary stents, silicone voice prostheses and acrylic dentures (Costerton *et al.*, 1999). One of the bacterial species found to form mixed biofilms with *C. albicans* is *Staphylococcus epidermidis* (Adam *et al.*, 2002).

4.1.2 – Biofilm properties

Microorganisms growing within biofilms have a number of properties that clearly distinguish them from planktonic populations. These include:

- Protection, where the biofilm structure or phenotype protects microorganisms from host defences and predators, from microbial agents (slow growth rate, poor penetration and altered phenotype), from desiccation and from fluid hydrodynamic and mechanical forces;
- Differences in phenotypic expression and growth characteristics;
- Competition and exchange of nutrients affecting availability of nutrient acquisition: elevated concentrations of nutrients, microbial and environmental heterogeneity for metabolic cooperation, spatial heterogeneity to optimize transport of by-products and increase nutrient influx;
- Intercellular communication: quorum sensing/density-dependent communication and interspecies communication.

4.1.3 – Biofilm formation

According to Chandra *et al.* (2001), *Candida albicans* biofilm formation has been shown to proceed in three distinct development phases: early (0-11 h), intermediate (12-30 h) and mature (38-72 h) (Figure 4.3).

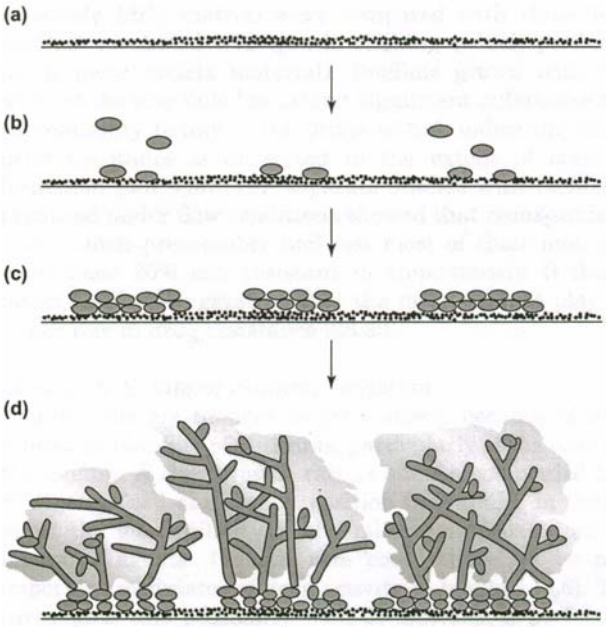


Figure 4.3 – Stages of *Candida* species biofilm formation. (a) inert surface; (b) initial yeast adhesion to the surface; (c) formation of the basal layers of yeast microcolonies; (d) mature biofilm containing hyphae and exopolymeric matrix. Adapted from Douglas (2003).

Biofilm formation is a step after adhesion, but although both phenomena involve cells and materials the methods used to quantify the cells must be different once the biofilm is more than a monolayer. So, the direct microscopic methods are not applicable in this case, with the exception of confocal scanning laser microscopy (CSLM) (Chandra *et al.*, 2001). CLSM has a number of significant advantages in biofilm research, as the fact that the sample may be examined in its natural hydrated state, avoiding problems of fixation and dehydration and because of its narrow depth of focus, thin optical sections can be recorded across biofilm profile (Wimpenny and Colasanti, 1997). The non-direct methods involve techniques that allow a quantification of the total biomass, as dry weight (Kuhn *et al.*, 2002b), by CFU determination (Jin *et al.*, 2004), crystal violet staining (Li *et al.*, 2003) or quantification of the cellular activity, by ATP determination (Jin *et al.*, 2004) or formazan salts formation (Kuhn *et al.*, 2002b).

Crystal violet staining (Stepanovic *et al.*, 2000) is one of the more expedite method to quantify biofilm biomass and can be used directly, without disrupting the biofilm. The crystal violet dye was first used by Christensen *et al.* (1985) to stain bacteria adhered to polystyrene tubes or plates. This method suffered some alterations that resulted in the elution of the dye from the adherent microorganisms (Merritt *et al.*, 1998). The cells previously immersed in methanol, allowing its fixation to the coupon, are stained with crystal violet (cationic dye) and after washing the coupons, the dye that bound to

the adherent cells is resolublized with acetic acid. The indirect biomass quantification is determined through the absorbance of the resulting acetic acid solution. The crystal violet staining has been used as indicator of the total attached biomass either in bacterial (Pitts *et al.*, 2003) or fungal (Li *et al.*, 2003) biofilms. A stain such as crystal violet is suitable for measuring the amount of biofilm, but not its activity. For that, formazan salts formation assays can be used.

The assays involving the use of tetrazolium salts evolved, since the early 1980s, with the advent of the 3-(4,5-dimethylthiazol-2-thiazyl)-2,5-diphenyltetrazolium bromide, also known as MTT (Mosmann, 1983). Roehm *et al.* (1991) described a substitute of MTT, XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyalamino)carbonyl]-2H-tetrazolium hydroxide} that was proved especially useful, once the water solubility of its formazan product enabled the simplification of the assay procedure. XTT is a yellow salt that is converted to dark blue formazan in the presence of metabolic activity; the mechanism of conversion of XTT to formazan includes mitochondrial succinoxidase and cytochrome P450 systems, as well, as flavoprotein oxidases (Altman, 1976). Tetrazolium salts are heterocyclic organic compounds that substitute the natural final acceptor (oxygen) in the biological redox process and are reduced to formazan derivatives by receiving electrons enzymically from substances of the hydrogen transport system or nonenzymically from artificial electron transporters, which enhance the reaction (Meletiadis *et al.*, 2001a). The more common electron-coupling agents used together with XTT are phenazine methosulfate (PMS) or menadione. One important point in using this component in biofilm research, is the fact that the formazan salts are water soluble allowing the study of intact biofilms as well as examination of biofilm drug susceptibility without disruption of biofilm structure.

Biofilms can be studied either in batch or continuous mode, using a variety of biological reactors. The batch model can be achieved either using reactors or coupons of the substratum material in well plates or also microtiter plates (ELISA), with the formation of the biofilm occurring in the bottom of the well. Among the various adhesion/biofilm model systems, the multi-well plate system permits rapid processing of large number of samples and on account of that has been the most used.

The flow conditions favour the development of extensive biofilms. *C. albicans* biofilms formed under static conditions contained small amounts of exopolymeric (EP) matrix.

By contrast, when the cells were incubated with shaking during biofilm formation a great production of EP matrix was observed (Hawser. *et al.*, 1997). It has also been noted that bacterial biofilms form more readily and are stronger when the cells are growing in high shear conditions (Donlan and Costerton, 2002).

Although the *Candida albicans* is the *Candida* species that form the biofilm with higher mass (Calderone, 2002c), within the non-*Candida albicans Candida* species there are some that have the capability of forming biofilms, as *C. parapsilosis* (Pfaller *et al.*, 1995) and *C. dubliniensis* (Ramage *et al.*, 2001). But for example *C. glabrata*, despite the fact of being capable of adhering to some surfaces (Hawser *et al.*, 1997; Kumamoto, 2002), did not exhibit EP matrix (Kuhn *et al.*, 2002b).

4.1.4 – Aims

Biofilms are the most common mode of microbial growth in nature and are also important in clinical infections, especially due to the high antibiotic resistance associated with them. In order to understand the influence of antifungal agents in biofilms it is important to study the mechanism and the factors that influence biofilm formation. The study of fungal biofilms is also of most importance due to the fact that in contrast to the extensive literature in bacterial biofilms (Merritt *et al.*, 1998; Pitts *et al.*, 2003) little attention has been paid to medically relevant fungal biofilms.

The present chapter describes two different approaches that were experimentally used in biofilm studies: one method that allows the determination of the total biomass, involving staining by crystal violet and another that quantifies the amount of active cells, based on tetrazolium salts formation.

In order to observe the influence of artificial saliva in the biofilm formation, biofilms were formed either in SDB (used as a control) and artificial saliva growth medium.

4.2 – Materials and Methods

4.2.1 – Media

Cells were grown in Sabouraud dextrose broth (SDB - Merck), that is the most common medium used to grow *Candida* species, or in an artificial saliva growth medium.

Sabouraud dextrose medium

The yeast cells were maintained in Sabouraud dextrose agar (SDA) that was prepared according to the manufacturer's instructions (30 g l^{-1}) plus 1.7 % of agar (Merck). Sabouraud dextrose broth (SDB – Merck), which was prepared using 30 g l^{-1} in water, was used to grow the cells in liquid medium.

Artificial saliva medium

In some experiments artificial saliva was used to mimic the *in vivo* oral conditions. This saliva was prepared according to Gal *et al.* (2001) with the following composition in mg l^{-1}: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5 KH_2PO_4, 200.0 Urea, 763.2 $Na_2SO_4.10H_2O$, 178.0 NH_4Cl, 227.8 $CaCl_2.2H_2O$ and 630.8 $NaHCO_3$. In order to have an artificial saliva growth medium glucose (2 g l^{-1}), yeast extract (2 g l^{-1}) and peptone (5 g l^{-1}) were added to the solution described (Johnson *et al.*, 2000). The pH was adjusted with carbon dioxide to 6.8.

4.2.2 – Yeast cells

The *Candida* species used were *Candida albicans* and *Candida dubliniensis* and two different strains of each species were assayed. In the case of *Candida albicans* one strain was from American Type Culture Collection, ATCC 32354 (*Candida albicans* B311) and the other was a clinical isolate (*Candida albicans* 12A). Regarding *Candida*

dubliniensis, the two strains were obtained from CBS (*Candida dubliniensis* 7987 and *Candida dubliniensis* 7988).

For all the assays, yeast cells were grown for 24 h in SDA at 37 °C. The cells were then inoculated in SDB for 18 h at 37 °C and 150 rpm. After incubation cells were harvested by centrifugation for 10 min at 5000 rpm and 4 °C.

4.2.3 – Acrylic surfaces

The acrylic coupons were prepared as described by Samaranayake and MacFarlane (1980). Briefly, 1.5 g of self-polymerizing acrylic powder was mixed with 1 ml of the monomer in liquid phase and after mixing the solution was poured onto a surface covered with aluminium foil. After 45 s another surface was placed on top of the polymerizing mixture. The acrylic sheet, polymerized during 30 min, was cut into 8 × 8 mm^2.

4.2.4 – Biofilm formation

Yeast cells were sub-cultured in Sabouraud dextrose agar for 24 h at 37 °C followed by growth in Sabouraud dextrose broth (SDB) at 37 °C and 150 rpm for 18 h. Cells were harvested by centrifugation (5000 rpm, 10 min) and resuspended in SDB or in artificial saliva growth medium to 5×10^7 cells ml^{-1}. The biofilm was formed on acrylic coupons (8×8 mm^2) in 24 well plates; each well containing 1 ml of the yeast cells suspension. The medium (SDB or artificial saliva growth medium) was replaced by fresh medium each 12 h and the biofilm was analysed after 7, 14, 24, 48 and 72 h of formation.

Crystal violet

The coupons containing the biofilm were removed from each well and immersed 15 min in new well plates containing 1 ml of methanol in each well. After withdrawing the methanol, the coupons were allowed to dry at room temperature before adding 600 µl of crystal violet to each well. After 5 min the coupons were washed in water and

immersed in acetic acid (33 %) to dilute the stain. The absorbance was then read at 570 nm.

Tetrazolium salts

The coupons containing the biofilms were withdrawn from each well and immersed in 1 ml of a solution of 100 μg μl^{-1} of XTT and 10 μg μl^{-1} of PMS. The well plate was incubated in the dark for 3 h with agitation (150 rpm). Each solution was centrifuged for 3 min at 1000 rpm and the absorbance was read at 490 nm.

4.3 – Results

To mimic the oral conditions the biofilms were formed in artificial saliva. SDB medium was used to grow biofilms as a control, since it is the most appropriate medium for *Candida* species growth. The artificial saliva growth medium used in this case had some additional components, as glucose, peptone and yeast extract in order to allow the yeast growth and thus to enable biofilm formation.

While in the case of the adhesion studies the method used for cell enumeration was the microscopic observation, in the case of biofilm mass quantification this is not possible due the its composition (cells plus EP matrix) and structure. So, the biofilm quantification was obtained using two different approaches, one that quantifies the total biomass formed, using crystal violet staining and another that quantifies the amount of active cells using a tetrazolium salt (XTT).

Before analyzing the biofilm formation these methods were optimized in order to achieve more accurate and trustful results.

4.3.1 – Methods optimization

In order to test if the crystal violet method is able to accurately assess the amount of cells present, the absorbance of a cell suspension treated with crystal violet was compared with that of non stained cells measured at 620 nm (Figure 4.4).

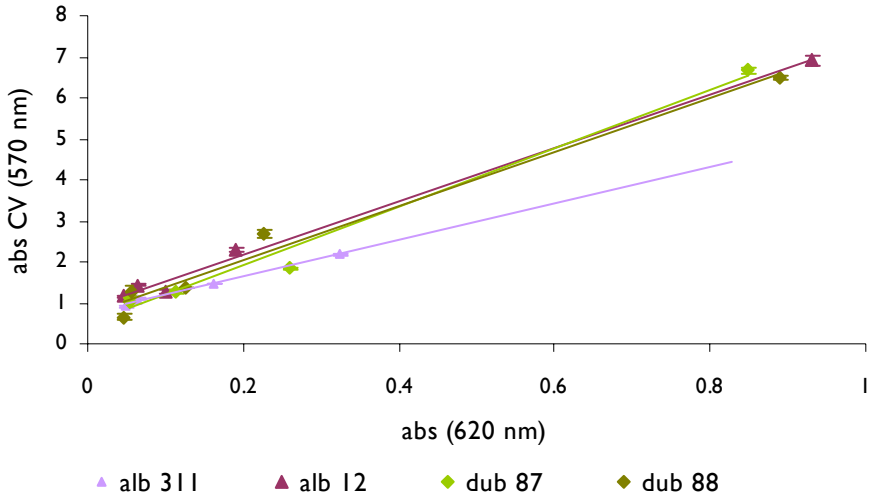
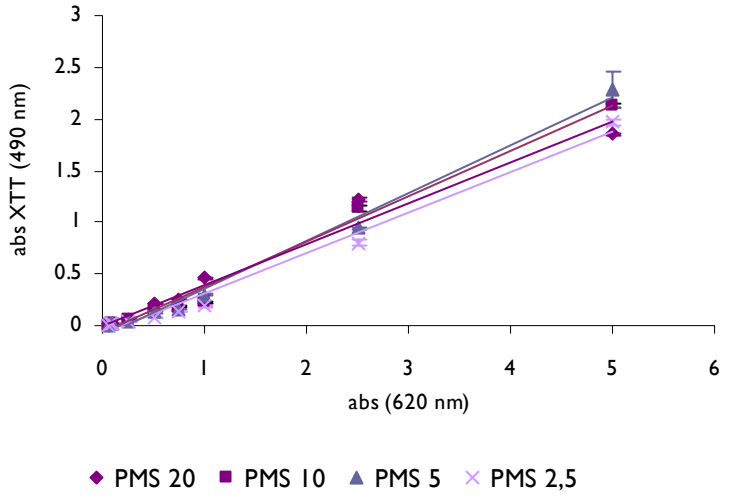


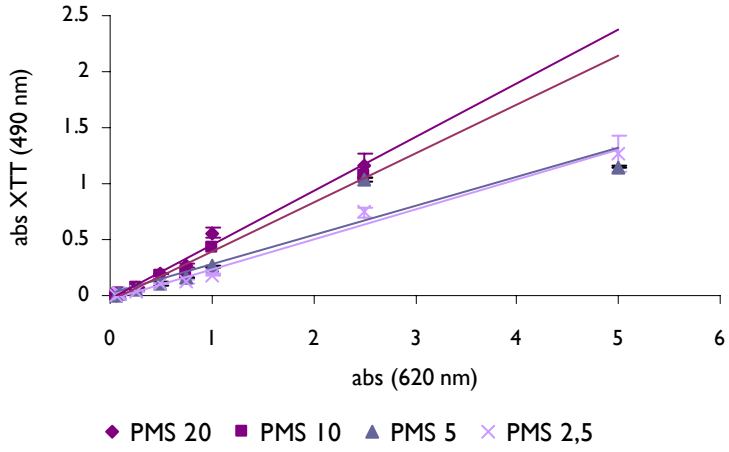
Figure 4.4 – Absorbance of non stained suspensions of *Candida albicans* B311 (albB311 – r^2=0.995, p=0.003), *Candida albicans* 12 A (alb12 albB311 – r^2=0.985, p=0.001), *Candida dubliniensis* 7987 (dub 87 – r^2=0.955, p=0.000) and *Candida dubliniensis* 7988 (dub 88 – r^2=0.981, p=0.001) versus the absorbance of the corresponding cell suspensions stained by crystal violet.

Figure 4.4 shows a linear correlation and similar slopes for all the strains. The correlation factor obtained in all cases was statistically significant, which confirms that crystal violet (CV) staining can be adequate to quantify cells.

As it was described in the introduction, the XTT assay needs an electron-coupling agent, which in this case was PMS. Thus, the optimal concentration of the pair XTT and PMS was determined. This determination was done for one strain of each species: *Candida albicans* 12A (Figure 4.5) and *Candida dubliniensis* 7987 (Figure 4.6).

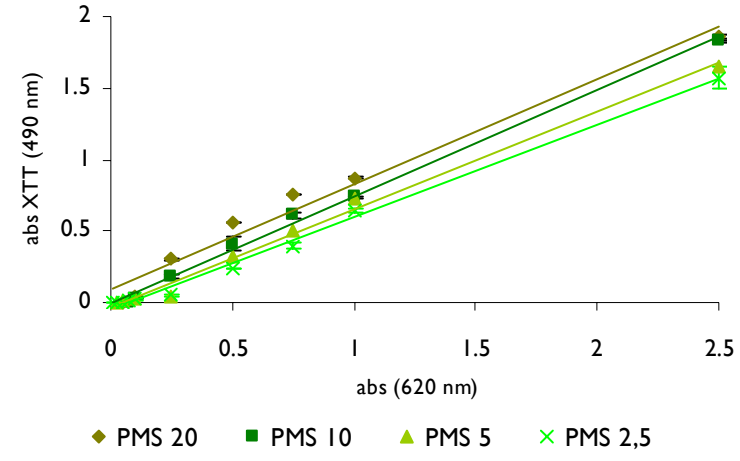


(a)

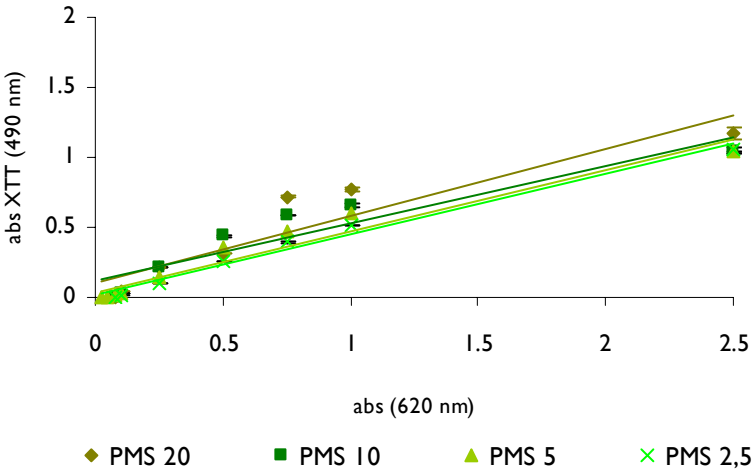


(b)

Figure 4.5 – Relation between the absorbance of a cell suspension of *Candida albicans* 12A treated with 100 µg/ml (a) and 50 µg/ml (b) of XTT and 20 µg/ml, 10 µg/ml, 5 µg/ml and 2.5 µg/ml of PMS and the absorbance at 620 nm, of a non-treated cell suspension. The p value is equal to 0.000 in all cases.



(a)



(b)

Figure 4.6 – Relation between the absorbance of a cell suspension of *Candida dubliniensis* 7987 treated with 100 µg/ml (a) and 50 µg/ml (b) of XTT and 20 µg/ml, 10 µg/ml, 5 µg/ml and 2.5 µg/ml of PMS and the absorbance at 620 nm, of a non-treated cell suspension. The p value is equal to 0.000 in all cases.

In order to simplify the selection of the better pair of XTT/PMS concentrations, the values of correlation factors obtained for each combination are presented in Table 4.1.

Table 4.1 – Correlation factors (r2) obtained for the variation of XTT and PMS absorbence of suspensions of *Candida albicans* 12 A and *Candida dubliniensis* 7987

XTT	PMS	Correlation factors (r^2)	
		Candida albicans 12 A	*Candida dubliniensis* 7987
100	2,5	0.985	0.996
	5	0.989	0.992
	10	0.989	0.996
	20	0.978	0.975
50	2,5	0.988	0.983
	5	0.895	0.943
	10	0.990	0.876
	20	0.986	0.869

The significance value (p) is not presented in Table 4.1 once it is null for all cases. From Figures 4.5 and 4.6 and from Table 4.1 it is possible to conclude that the better correlation factor is obtained for 100 µg ml^{-1} of XTT and 10 µg ml^{-1} of PMS. So this was the selected combination of the two compounds.

The relation between the absorbance of cell suspensions stained by XTT and of standard planktonic cells (620 nm) was determined and is presented in Figure 4.7.

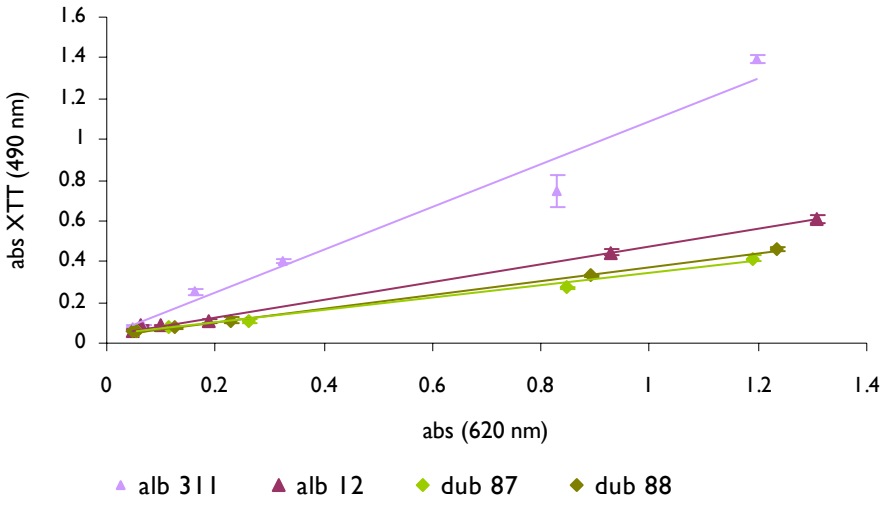


Figure 4.7 – Absorbance of non stained suspensions of *Candida albicans* B311 (albB311 – r^2=0.995, p=0.003), *Candida albicans* 12 A (alb12 albB311 – r^2=0.985, p=0.001), *Candida dubliniensis* 7987 (dub 87 – r^2=0.955, p=0.000) and *Candida dubliniensis* 7988 (dub 88 – r^2=0.981, p=0.001) versus the absorbance of the corresponding cell suspensions stained by XTT.

Observing Figure 4.7 it is possible to see that for all strains, with the exception of *Candida albicans* B311, similar slopes were obtained as well as correlation factors.

The values of the absorbance of CV and XTT resulting solutions were also correlated with each other for each strain. Table 4.2 presents the values of the correlation factor and respective significance values for each strain.

Table 4.2 – Correlation factors (r^2) and significance values (p) obtained for the absorbance of CV stained suspensions of *Candida albicans* B311, *Candida albicans* 12 A, *Candida dubliniensis* 7987 and *Candida dubliniensis* 7988 versus the absorbance of the same cell suspensions stained by XTT

Strain	r^2	p
Candida albicans B311	0.973	0.014
Candida albicans 12A	0.991	0.000
Candida dubliniensis 7987	0.989	0.000
Candida dubliniensis 7988	0.978	0.001

The values in Table 4.2 show that for planktonic cells there is a statistical significant correlation between the absorbance of both evaluation methods for all the strains studied. It is also interesting to notice that *Candida albicans* B311 present a different behaviour from the others.

4.3.2 – Biofilm formation studies

The biofilm formation was carried out for 7, 14, 24, 48 and 72h with two strains of *Candida albicans* and of *Candida dubliniensis* on acrylic coupons, either in the presence of SDB or artificial saliva. Figures 4.8 and 4.9 present the evolution of the biofilm biomass quantified using crystal violet staining.

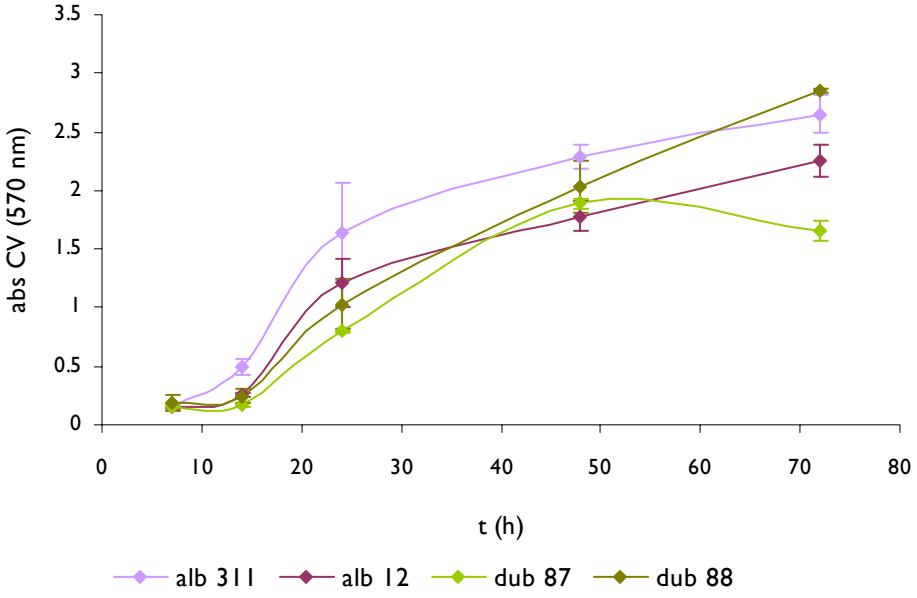


Figure 4.8 – Absorbance values of crystal violet solutions obtained from biofilms formed in SDB after 7, 14, 24, 48 and 72h by *Candida albicans* 12 A (alb12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88).

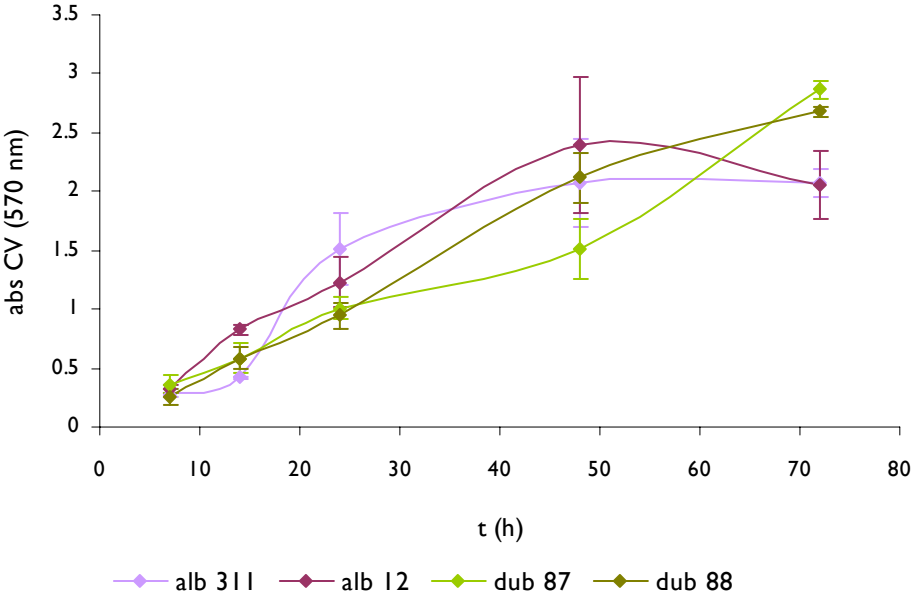


Figure 4.9 – Absorbance values of crystal violet solutions obtained from biofilms formed in artificial saliva after 7, 14, 24, 48 and 72h by *Candida albicans* 12 A (alb12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88).

It is interesting to notice that the biofilms of *Candida dubliniensis* presented different behaviours in both media. In SDB medium the biofilm mass of *Candida albicans* and *Candida dubliniensis* 7988 increased until 72h and *Candida dubliniensis* 7987 reached a *plateau* after 48 h (Figure 4.8), while in artificial saliva this behaviour was the opposite.

In fact, biofilms of *Candida albicans* and *Candida dubliniensis* 7988 presented a plateau after 48h, but for *Candida dubliniensis* 7987 the biofilm mass increased until 72h.
In order to help the interpretation of these results the slope between 48 and 72 hours of the biofilm formation profile was determined and the values are presented in Table 4.3.

Table 4.3 – Values of the slopes of the biofilm formation profiles (between 48 and 72 h) of *Candida albicans* B311, *Candida albicans* 12A, *Candida dubliniensis* 7987 and *Candida dubliniensis* 7988 evaluated with CV, in both biofilm formation media

	Slope	
Strain \ Medium	SDB	Artificial saliva growth media
Candida albicans B311	0.015	0.023
Candida albicans 12A	0.020	0.049
Candida dubliniensis 7987	-0.010	0.021
Candida dubliniensis 7988	0.034	0.049

As observed in Table 4.3, a negative slope was obtained for biofilms of *Candida dubliniensis* 7897 being this slope very small compared with the others and it can be considered almost zero, meaning that a plateau was achieved. In the case of saliva, positive and high slopes were obtained for biofilms of all the strains. It is also interesting to notice that the slopes of the biofilm formation profiles of *Candida albicans* B311 and *Candida dubliniensis* 7987 are similar and the same happens for the other two strains.

The biofilms activity profiles formed on either SDB or artificial saliva growth medium are presented in Figures 4.10 and 4.11

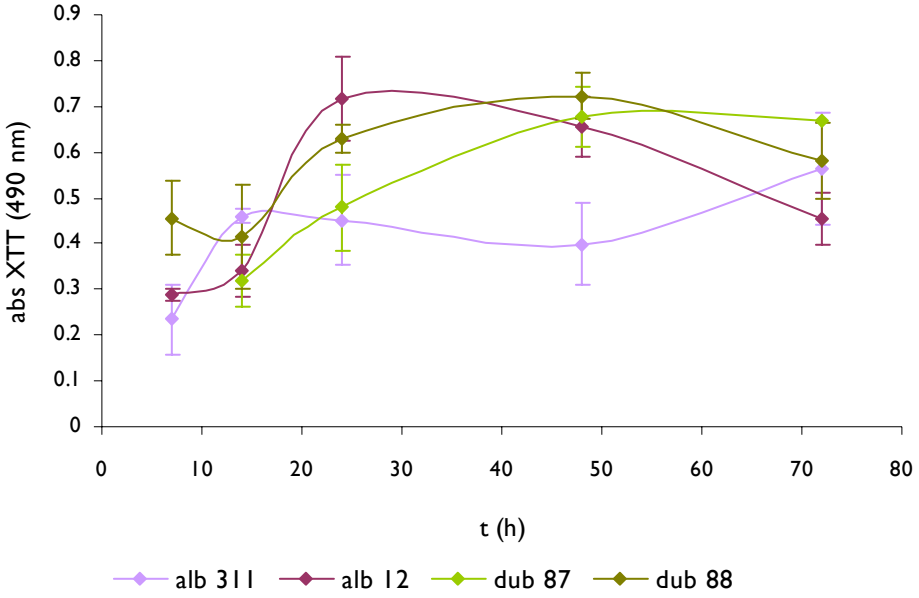


Figure 4.10 – Absorbance values of XTT solutions obtained from biofilms formed in SDB after 7, 14, 24, 48 and 72h by *Candida albicans* 12 A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88).

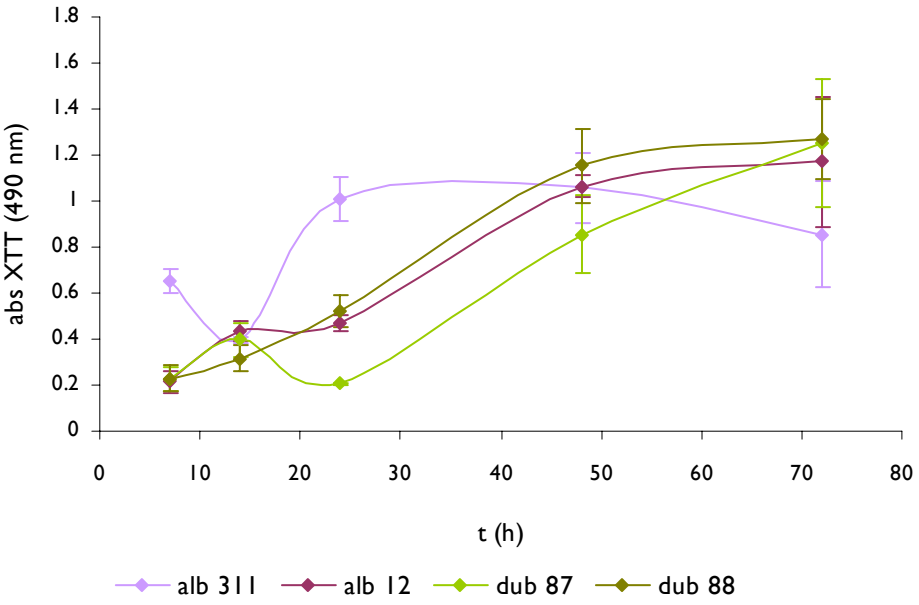


Figure 4.11 – Absorbance values of XTT solutions obtained from biofilms formed in artificial saliva after 7, 14, 24, 48 and 72h by *Candida albicans* 12 A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88).

As in the case of biofilm mass accumulation, the biofilm of *Candida dubliniensis* 7987 had a different behaviour from those of the other strains in terms of activity. In SDB, *C. dubliniensis* 7987 biofilms presented a plateau after 48h, but the activity of *Candida albicans* 12A and *Candida dubliniensis* 7988 biofilms decreased after that time. In the

case of artificial saliva, the cellular activity of *Candida dubliniensis* 7987 biofilms increased until 72 h, while the biofilms formed by the other two strains presented a plateau after 48h.

The slope of the different curves between 48 and 72 h was determined and is presented in Table 4.4.

Table 4.4 – Values of the slopes of the biofilm formation profiles (between 48 and 72 h) of *Candida albicans* B311, *Candida albicans* 12A, *Candida dubliniensis* 7987 and *Candida dubliniensis* 7988 evaluated with XTT, in both biofilm formation media

	Slope	
Strain \ Medium	SDB	Artificial saliva growth media
Candida albicans B311	0.007	-0.008
Candida albicans 12A	-0.008	0.004
Candida dubliniensis 7987	-0.000	0.016
Candida dubliniensis 7988	-0.006	0.005

The profiles of all *Candida* strains biofilms formed in SDB presented very small or null slopes between 48 and 72h. So, it can be considered that cell biofilm activity achieves a plateau after 48h. Regarding biofilm profiles formed in artificial saliva the slope of *Candida albicans* B311 is negative, contrary to the behaviour in the other medium, but it is also very small. The only biofilm evolution profile that does not present a null slope is the one formed by *Candida dubliniensis* 7987.

In order to allow a better comparison between the media used (artificial saliva and SDB), Table 4.5 presents the significance values obtained by both quantification methods (CV and XTT).

Table 4.5 – Significance values (p) obtained comparing biofilms formed by *Candida albicans* B311 (alb 311), *Candida albicans* 12 A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88) in SDB and artificial saliva growth medium, for both evaluation methods and all the times assayed

	Significance value (p)							
	CV				XTT			
Tempo (h)	alb 311	alb 12	dub 87	dub 88	alb 311	alb 12	dub 87	dub 88
7	0.005	0.009	0.041	0.382	0.009	0.142	0.002	0.016
14	0.229	0.003	0.005	0.006	0.055	0.157	0.325	0.253
24	0.683	0.922	0.460	0.571	0.002	0.038	0.020	0.137
48	0.384	0.279	0.067	0.317	0.003	0.001	0.550	0.034
72	0.007	0.305	0.026	0.481	0.122	0.000	0.001	0.003

The red values, in Table 4.5, represent the p values under 0.05, meaning that there are significative differences with a 95% confidence level. In the case of biomass quantification the major differences appear in the beginning of the biofilm formation, while in the case of activity these differences are more notorious after 24h.

Comparing the results obtained in terms of biofilm mass and biofilm activity it can be concluded that cell activity is not dependent on cell number. In the case of a mature biofilm, the number of total cells might be high but their activity can be low. For instances, the cells in the deeper layer can be less active, due to diffusional limitations.
The evolution of the ratio absorbance of XTT solution/absorbance of CV solution (XTT/CV) along the times assayed during biofilm formation, is presented in Figure 4.12.

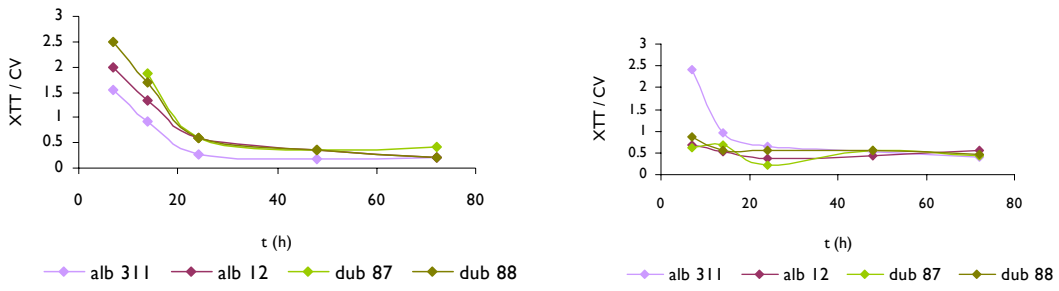


Figure 4.12 – Ratio "absorbance of XTT solution / absorbance of CV solution" (XTT/CV) versus time for biofilms formed in SDB (a) or artificial saliva (b) by *Candida albicans* B311 (alb 311), *Candida albicans* 12A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88).

Figure 4.12 shows that for both media the ratio XTT/CV is high in the beginning but decreases significantly after 24h, when the biofilm becomes more mature. It means that the biofilm activity decreases along the time. It is interesting to note that this behaviour is similar for all the strains, with the exception of biofilms of *Candida albicans* B311, which presents a higher value of XTT/CV after 7h of formation in the case of artificial saliva.

Figure 4.13 presents SEM images of 48h biofilms of all the strains in SDB.

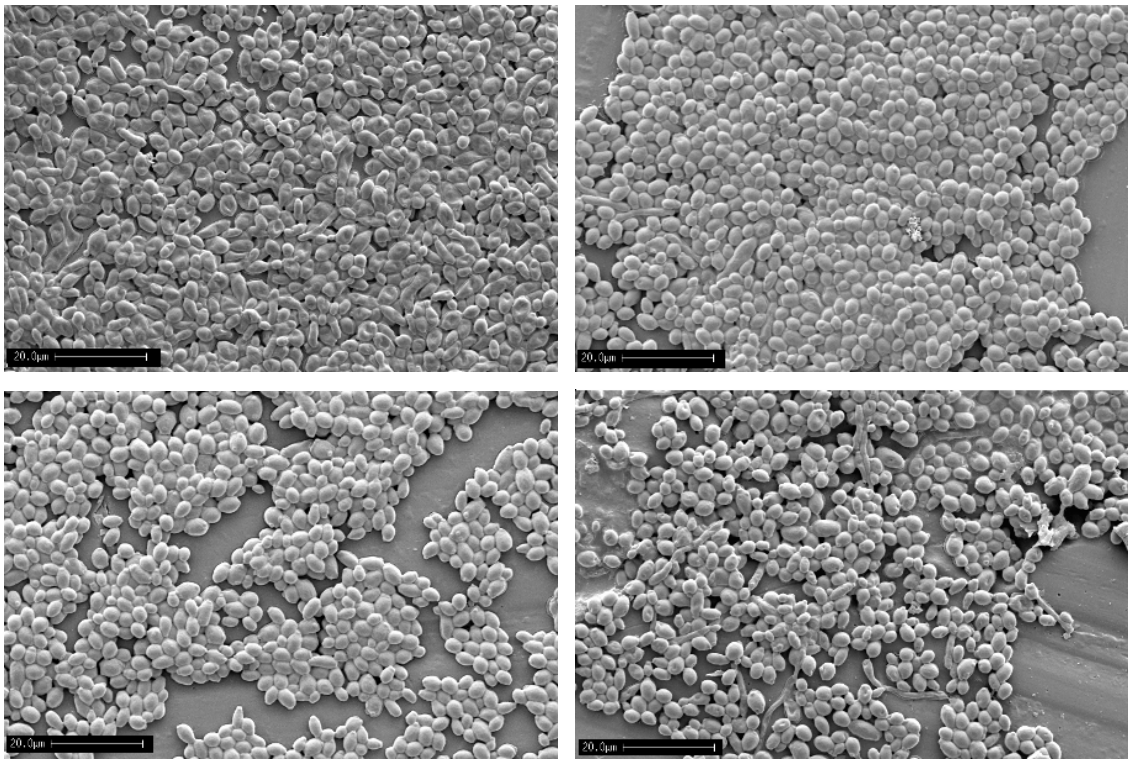


Figure 4.13 – SEM images (1000X) of biofilms formed in SDB after 48h for *Candida albicans* B311 (a), *Candida albicans* 12 A (b), *Candida dubliniensis* 7987 (c) and *Candida dubliniensis* 7988 (d). In the images the bar represents 20 μm.

As shown by Figure 4.13, with a 1000 X magnification all the biofilms are compact and it is possible to observe some pseudohyphae in some samples. In order to better visualize pseudohyphae structures in biofilm formed in saliva, the observations were made in parts of the biofilm presenting a low thickness. Actually this formation is easily found in the presence of saliva and with a higher magnification (3000 X) as it is presented in Figure 4.14.

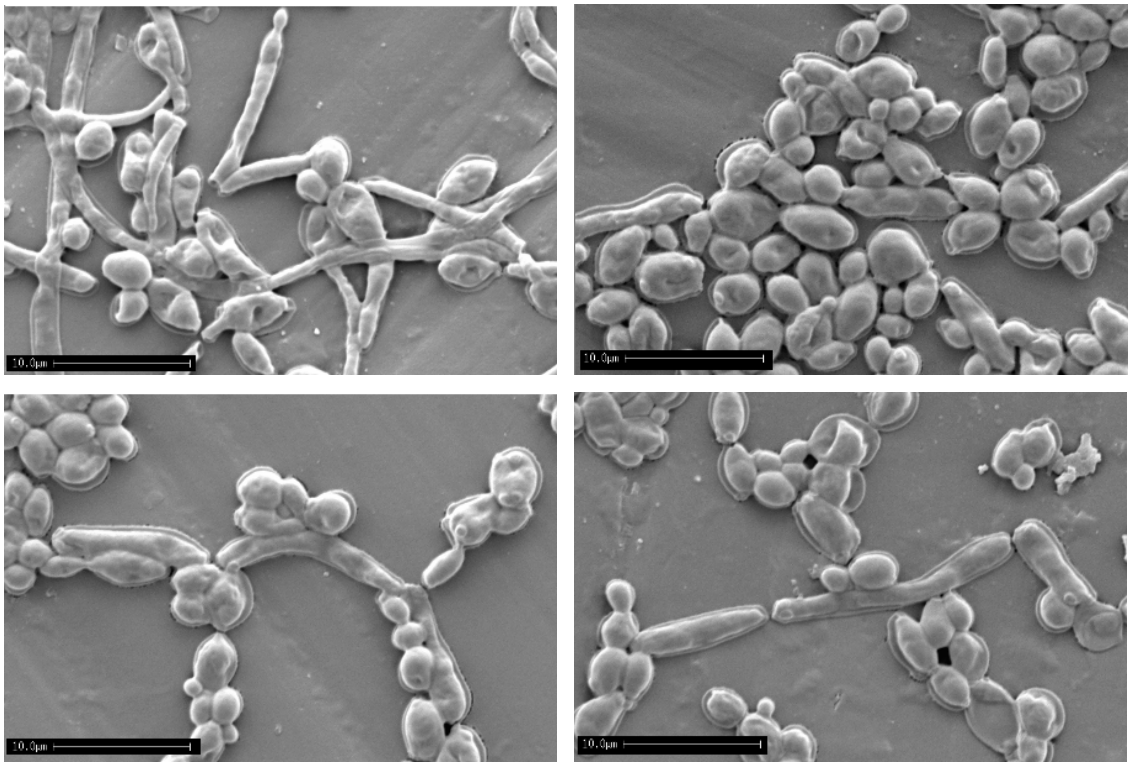


Figure 4.14 – SEM images (3000X) of biofilms formed in artificial saliva after 48h by *Candida albicans* B311 (a), *Candida albicans* 12 A (b), *Candida dubliniensis* 7987 (c) and *Candida dubliniensis* 7988 (d). In the images the bar represents 20 μm.

Undoubtedly, *Candida albicans* B311 is from all the strains, the one that forms biofilms with more and longer pseudohyphae, both in SDB and in artificial saliva. All the other strains form biofilms where the presence of pseudohyphae is only noticed in artificial saliva growth medium.

4.4 – Discussion

The first part of this work included the optimization of the methods used in the evaluation of Candidal biofilms biomass and activity. Concerning the optimal concentrations of XTT and PMS, the values obtained in this work (100 μg ml^{-1} of XTT and 10 μg ml^{-1} of PMS) are in the range of the ones described by other authors (Hawser *et al.*, 1998; Meletiadis *et al.*, 2001b). This method is usually used in studies of the susceptibility of *Candida* species biofilms to antifungal agents (Hawser *et al.*, 1998; Meletiadis *et al.*, 2001b). However, Kuhn *et al.* (2003) described some limitations of this

method. They conclude that it cannot be assumed that there is necessarily a linear relationship between the number of cells and the colorimetric signal and that the relationship between XTT concentration used and the resultant colorimetric signal is not necessarily proportional. These limitations could be easily overcome if for the microorganisms under study and the conditions used it would be possible to establish a linear relation between the number of cells and the XTT signal. The high values of the correlation factors (Table 4.1) between the absorbance of XTT and CV solutions and the absorbance of planktonic cells are high meaning that these methods can be applied for cell quantification of suspended cultures at the exponential growth phase. A good correlation between the absorbance of CV and XTT solutions allows the use of both methods to compare differences in cell concentrations. Therefore they can be applied to biofilms. However, care must be taken because cells in the biofilm present different metabolic activities and so it is expected to have different signals from those obtained in a suspended culture with the same cellular density. So, XTT can only evaluate biofilm activity. In the present work the correlation was statistically significative ($p<0.05$ in all cases).

One of the main conclusions of this part of the work is the fact that *Candida* species of *C. albicans* and *C. dubliniensis* can form mature biofilms, in opposition, for instances, to *Saccharomyces cerevisae* that can adhere but not form biofilm (Chandra *et al.*, 2001). This fact is very important once it is one of the pathogenic features of *Candida* species. As it was described before biofilm formation includes several phases, such as growth, proliferation and maturation. From the analysis of Figures 4.8 to 4.11 it is possible to verify that all the biofilms formed presented those phases, in spite of the differences found between the strains.

Another conclusion is that biofilm formation is either strain or species dependent. *Candida dubliniensis* 7987 presents always a different behaviour compared with the other strain of the same species and the strains of *C. albicans*. *Candida albicans* 12A and *Candida dubliniensis* 7988 have a similar behaviour in almost all conditions studied, with the exception of biofilm biomass formation in artificial saliva. *Candida albicans* B311 also shows a different behaviour from the other strains in all assays. The results obtained

concerning the biofilm activity of *Candida dubliniensis* 7987 are similar to the ones obtained by Ramage *et al.* (2001).

Comparing both methods of biofilm evaluation, it is interesting to notice that although the biomasses increase along the time their activity decrease. This means that although the amount of biomass present in the biofilm is high its activity is low, which can be explained by the fact that a biofilm is composed by several cell layers and maybe the basal ones are not so active as the ones on the top of the biofilm. This happens for all the strains studied. The ratio between the activity and the biomass was determined for biofilms formed in artificial saliva and SDB. From the analysis of Figure 4.12 it is possible to observe that after 24h this ratio decreases for all the strains. This fact is in accordance with what was stated above that relates the increase in biomass with a decrease in activity.

Yin *et al.* (2003) reported that cell activity measured by XTT is linearly associated with the number of cells, confirming the reliability of XTT staining in biofilm activity quantification. However, as biofilm cells are enclosed in an EP matrix, this may possibly impose a limited access to nutrients and oxygen, resulting in possible alterations in cellular metabolic activity. If this is the case, the XTT assay, which is based on metabolic activity, may not determine accurately the number of cells. Other method, as CV staining should be used to quantify the total biomass. Comparing to dry weight or CFU evaluation, the CV staining has the advantage of being used directly in the biofilm, with no need to remove it from the surface.

Pseudohyphae formation has been considered as one of the virulence factors of *Candida* species and one of the responsible for adhesion interactions (Brown, 2002b). The present results (Figure 4.14) demonstrated that artificial saliva promotes the formation of pseudohyphae, but no evidence was displayed that pseudohyphae enhance biofilm formation.

Comparing both media, it is interesting to note that *C. dubliniensis* 7987 biofilm formation profiles present a positive and significant slope in the case of artificial saliva but the opposite happens in the case of SDB, for both methods of biofilm evaluation. From Table 4.4 it can be observed that the significative differences between both media are not constant, they vary with the evaluation method, strain and biofilm age.

The artificial saliva was supplemented with carbon sources to facilitate biofilm growth and because carbon sources are also present in the oral cavity. Concerning biofilm formation in artificial saliva growth medium, there was an increase in the activity and no alteration in biomass amount.

Other studies involving the use of artificial saliva resulted in different conclusions. According to Jin *et al.* (2004) the presence of saliva did not significantly influence biofilm formation, while according to Nikawa *et al.* (1997) the presence of saliva increased biofilm formation of *C. albicans*. It is well known that some salivary proteins, such as human fibronectin and members of the praline-rich protein family, can act as receptors for *C. albicans*. On one hand, these proteins, when immobilized on a surface, may possibly promote *Candida* adhesion and subsequent biofilm formation by acting as receptors for free-living planktonic yeasts whilst on the other, they may simultaneously block binding sites originally present on the substratum. In addition, the presence of salivary antimicrobial proteins has been demonstrated (San Millan *et al.*, 2000) which further complicates this issue (Jin *et al.*, 2004). In the present study the influence of saliva on biofilm formation was assessed using artificial saliva growth medium instead of natural saliva. As described in Chapter 2, the use of whole saliva has some disadvantages, including the variations with the donor and the time of the day, which can be overcome with an artificial solution were the parameters are always reproducible. Another advantage of the use of artificial saliva in this work is the study of the direct influence of parameters as the effect of salts and pH, which is not possible with whole saliva that possesses proteins and other specific components.

Considering the number of initially adhered cells to acrylic (Chapter 2), the strains studied presented no statistical differences compared to each other, either in water or artificial saliva. However, as biofilm formation is concerned, *Candida dubliniensis* 7987 presented a different behaviour from *Candida albicans* and *Candida dubliniensis* 7988, the latter two behaving similarly. This difference was obtained both in terms of biomass accumulation and cellular activity and for both media (SBD and artificial saliva growth medium). As a general conclusion, the number of initially adhered cells had no direct correlation with biofilm evolution and thus adhesion and biofilm formation can be considered distinct factors of Candidal virulence. Additionally biofilm formation was shown to be strain dependent.

Chapter 5 – EFFECT OF SUBINHIBITORY CONCENTRATIONS OF ANTIFUNGAL AGENTS

5.1 – Introduction

During the past two decades the frequencies and the types of life-threatening fungal infections have increased dramatically in immunocompromised patients (Georgopapadakou and Walter, 1996). Among the factors that have contributed to that are:

- The expansion of severely ill and/or immunocompromised patient populations with human immunodeficiency virus (HIV) infection, with chemotherapy-induced neutropenia, and receiving organ transplant-associated immunosuppressive therapy;
- More invasive medical procedures, such as extensive surgery and the use of prosthetic devices and vascular catheters;
- Treatment with broad-spectrum antibiotics or glucocorticosteroids
- Peritoneal dialysis or hemodialysis.

These fungal infections are usually prevented or treated with antifungal agents, as fluconazole and amphotericin B. As the use of these agents is increasing, some resistance is starting to appear and so the influence of these agents in the fungal virulence is under study.

5.1.1 – Antifungal agents

The systemic antifungal compounds that are currently in clinical use can be divided in four major classes: the polyene antibiotics, the azole derivatives, the allylamines and the morpholine derivatives.

Polyenes

The polyene antibiotics, produced by *Streptomyces* species are fungicidal and have the broadest spectrum of activity of any clinical useful antifungal compound. Among the most common polyenes are amphotericin B, nystatin and natamycin (Georgopapadakou and Walter, 1996). Amphotericin B (Figure 5.1), the most used polyene, can form soluble salts in both basic and acid environments, is not orally or intramuscularly absorbed and is virtually insoluble in water (Sanglard and Bille, 2002).

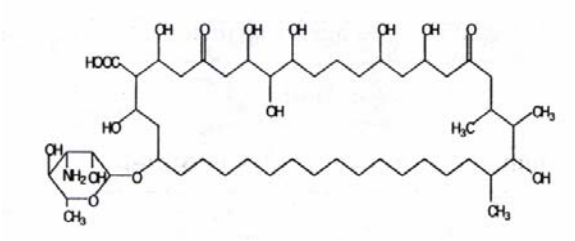


Figure 5.1 – Chemical structure of amphotericin B. Adapted from Sanglard and Bille (2002).

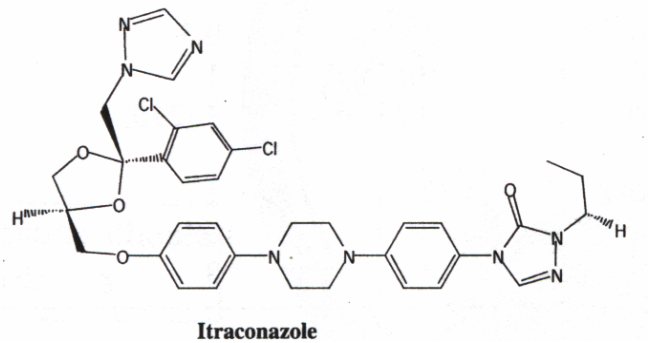
The polyenes complex the ergosterol in the plasma membrane, causing membrane disruption, increased permeability, leakage of cytoplasmatic content, and cell death (Bolard, 1986).

The advantage of amphotericin B is its much higher affinity for ergosterol-containing membranes than for cholesterol-containing membranes. Its acute and chronic side effects may be reduced in newer formulations, such as liposomes, lipid complexes and colloidal dispersions (Georgopapadakou and Walter, 1996).

Microbial resistance to polyenes is associated with altered membrane lipids, particularly sterols. Other mechanism of resistance may involve altered phospholipids and increased catalase activity with decreased susceptibility to oxidative damage (Georgopapadakou and Walter, 1996).

Azoles

The azole derivatives, discovered in the late 1960s, are totally synthetic and are the most rapidly expanding group of antifungal compounds. Azole antifungal agents used in medicine are categorized into N-1 substituted imidazoles (ketoconazole, miconazole, clotrimazole) and triazoles (fluconazole, itraconazole) (Figure 5.2) (Sanglard and Bille, 2002). Depending on the particular compound, azole antifungal agents have fungistatic, broad-spectrum activity that includes most yeasts and filamentous fungi.



Itraconazole

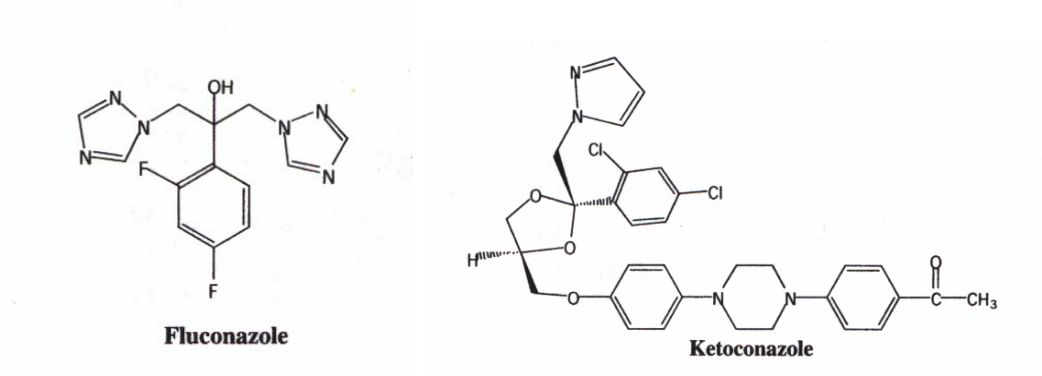


Figure 5.2 – Chemical structures of some azoles. Adapted from Sanglard and Bille (2002).

Azoles have a cytochrome P450 as a common cellular target in yeast or fungi. They act primarily on ergosterol biosynthesis at the C-14 demethylation stage, a three-step oxidative reaction catalyzed by the cytochrome P450 enzyme 14 α-sterol demethylase. Azole antifungal agents are generally free of serious toxicity; however rare cases of fatal hepatotoxicity have been reported, particularly with ketoconazole (Georgopapadakou and Walter, 1996).

Among the most used azoles is fluconazole that is metabolically stable, water soluble and has low lipophilicity and plasma protein binding. This agent is active by both oral and intravenous routes which have identical pharmacokinetics, i. e. once-daily dosing, high blood levels and rapid equilibration of drug in the body with good tissue distribution, including penetration into the cerebrospinal fluid are obtained.

Allylamines

These synthetic antifungal agents were discovered in 1970. The most common compounds of this class are Terbinafine and naftifine (Figure 5.3).

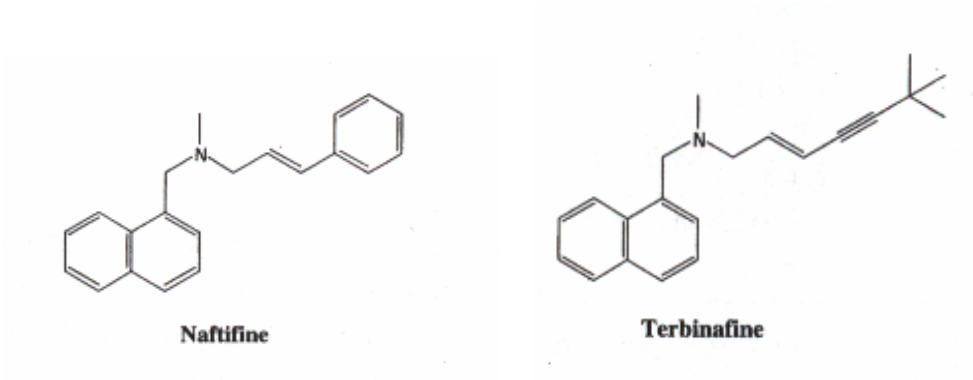


Figure 5.3 - Chemical structures of some allylamines. Adapted from Sanglard and Bille (2002).

Allylamines target squalene epoxidases, which is the first postsqualene enzyme of the ergosterols biosynthetic pathway.

Although terbinafine is fungicidal against dermatophytes and filamentous fungi (Arzeni *et al.*, 1998), it is only fungistatic against the majority of *Candida* species (Sanglard and Bille, 2002).

Morpholine derivatives

Included in this group are amorolfine (Figure 5.4), fenpropimorph and tridemorph.

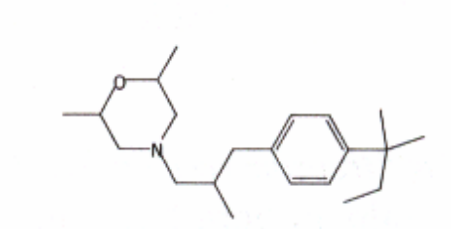


Figure 5.4 - Chemical structures of amorolfine. Adapted from Sanglard and Bille (2002).

These compounds are totally synthetic and, with the exception of amorolfine, are mostly used in agricultural fungicide preparations (Sanglard and Bille, 2002).

5.1.2 – Yeast targets for antifungal action

Different cellular processes involved in the biosynthesis of components required for growth of fungal cells have been targeted by antifungal agents. These targets are

specific for each antifungal agent and were described in the previous sub-section. Figure 5.5 depicts the general targets of the most relevant antifungal agents.

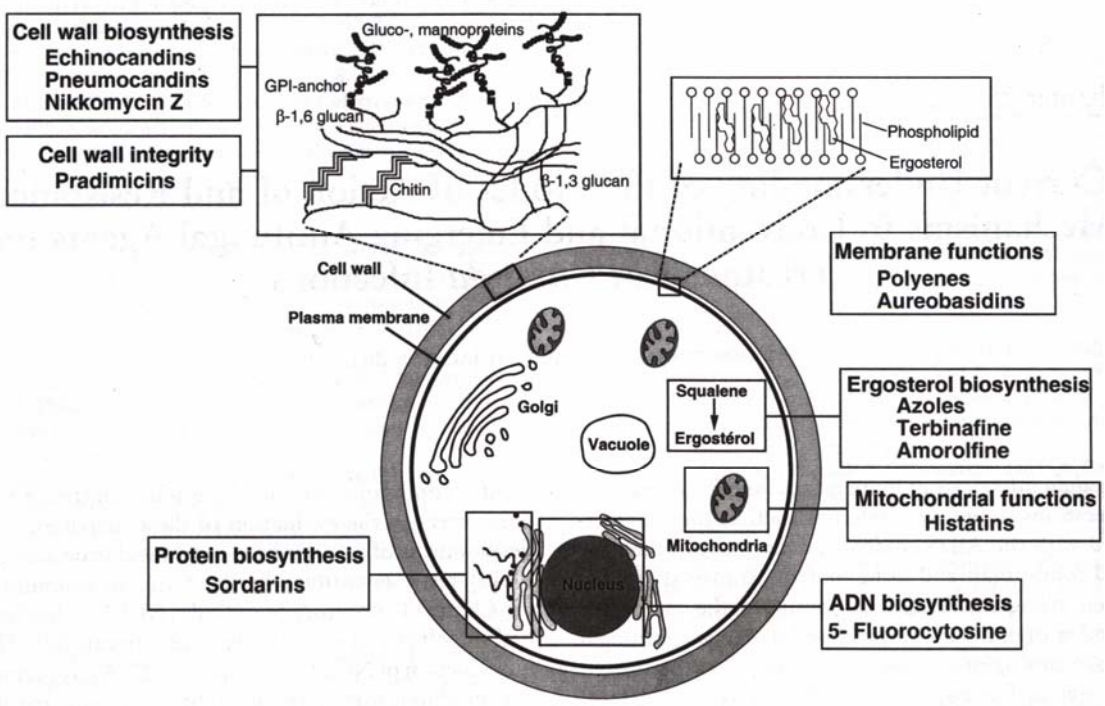


Figure 5.5 – Schematic view of a fungal cell and of the principal targets of antifungal agents. Adapted from Sanglard and Bille (2002).

The targets presented in Figure 5.5 include the cell membrane, the plasma membrane and other components of the cell envelope.

Candida cells, as a yeast cell, possess a cell envelope that comprises the plasma membrane, the periplasmatic space, the cell wall, and the fibrous layer associated with cytosol of the cell and the external environment (Bolard, 1991). Very few studies have been carried out on the plasma membrane of *C. albicans* compared to those on the cell wall. However, the main characteristic of the lipid composition, which differentiates *C. albicans* membranes from those of the human host, is well established: unlike the cholesterol of the host, the major sterol of *C. albicans* is ergosterol (Bolard, 1991).

5.1.3 – Resistance to antifungal agents

A successful clinical response to therapy typically not only depends on the susceptibility of the pathogenic organism but also relies heavily on the host immune

system, drug penetration and distribution, patient compliance, and absence of a protected or persistent focus of infection. Microbial resistance of a given pathogen must be regarded as a quantifiable variable, determined by measurement of drug susceptibility and must be defined with respect to a reference population (Sanglard and Bille, 2002). This quantifiable variable can be the minimal inhibitory concentration (MIC), which is the lowest drug concentration that avoids visible growth of the yeast; or the minimal fungal concentration (MFC), which is the lowest drug concentration that avoids visible growth of the yeast in subculture (Costa *et al.*, 1999). The MIC determination has been the goal of several studies and some methods have been proposed to improve the method approved by the National Committee for Clinical Laboratory Standards (NCCLS).

NCCLS M27 method

The introduction of standardized methods approved by the NCCLS for measuring MICs of a number of antifungal agents in selected fungal pathogens has been essential for interlaboratory comparisons and use in clinical and research laboratories. After passing through the stages of being a Proposed document (M27-P) in 1992 and a Tentative document (M27-T) in 1995, the NCCLS M27 methodology for testing yeasts became an Approved level document (M27-A) in 1997 (National Committee for Clinical Laboratory Standards, 1997). This method specifies inoculum size and preparation, test medium, incubation time and temperature, and end-point reading for flucytosine, amphotericin B, fluconazole, ketoconazole and itraconazole (Rex *et al.*, 2001). This method is based on a visual observation of cellular growth in RPMI medium supplemented with increasing concentrations of an antifungal agent. Although this method is standard it does not allow an exact determination of MIC, once it is based on visual observations.

In order to overcome this problem several quantitative methods have been developed, as the quantification of formazan salts formation and the rapid susceptibility assay (RSA). A semi-quantitative method was developed by PASCO Division of Becton-Dickinson.

Quantification of formazan salts

Colorimetric methods may be an alternative to the NCCLS visual method, since precise quantification of hyphal growth is achieved and clear-cut end points can be generate. Tetrazolium salts have been used as colorimetric indicators, since fungi convert them to coloured formazan derivatives, which can be quantified (Meletiadis *et al.*, 2001b). An assay was suggested by Meletiadis *et al.* (2000) involving the use of MTT (3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H tetrazolium bromide). The disadvantage of MTT is that the formazan derivatives formed are not water soluble (Meletiadis *et al.*, 2001a). An alternative method uses XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) that forms salts that are water soluble (Hawser *et al.*, 1998).

Rapid Susceptibility Assay (RSA)

This assay (Riesselman *et al.*, 2000) is based on the hypothesis that when susceptible fungi are exposed to an antifungal drug, their uptake of an exogenous substrate will be suppressed or inhibited. By measuring the amount of residual substrate in the medium compared to that for controls without drug, the susceptibility of a fungal isolate can be determined. One advantage of this method is that the results obtained are objective because the endpoint determinations are based in colorimetric differences, as assessed with a microplate reader. Moreover, MIC determinations obtained by evaluating substrate uptake are highly sensitive and should be more rapid than tests that rely on fungal growth.

PASCO

The PASCO Division of Becton-Dickinson developed a commercially available broth microdilution panel for the in vitro susceptibility testing of antifungal agents (Arthington-Skaggs *et al.*, 2000). For prototype testing, each panel contained eight antifungal agents, was prepared in advance and contained 10 serial two fold dilutions of each agent frozen in broth, together with positive and negative control wells. Advance preparation of the plates eliminated the need to prepare media and drug dilutions in-house and pipette prepared dilutions into individual microtiter plate wells. Another advantage of PASCO is that it includes quality control testing of each lot of plates.

5.1.4 – Subinhibitory concentrations of antifungal agents

As it was described before, Candidiasis is an important life-threatening fungal infection of immunocompromised patients and a common manifestation in patients with AIDS. The antifungal agents are used in these patients as a post-infection maintenance therapy. In this case, the dose of these agents is typically low, which may result in blood levels that are either clinically subinhibitory or less than the in vitro MIC (Hazen *et al.*, 2000). The exposure of patients to subinhibitory concentrations of antifungal agents may lead to alterations in the *Candida* cell properties. These alterations can affect the cell surface hydrophobicity (Hazen *et al.*, 2000), the adhesion process (Dorocka-Bobkowska *et al.*, 2003; Ellepola and Samaranayke, 1998; Zepelin *et al.*, 2002) and the ability for biofilm formation (Bachmann *et al.*, 2002; Kuhn *et al.*, 2002a).
In the mouth, the diluent effect of saliva and the cleansing action of the oral musculature often tend to reduce the viability of the agents to below that of the effective therapeutic concentration (Martin, 1989). Thus the organism in the oral cavity experiences only a limited exposure to the antifungal agent during treatment and the concentration of the drug may vary in different niches of the mouth (Ellepola and Samaranayke, 1998).

5.1.5 – Aims

The aim of the reported work was the study and comparison of adhesion and biofilm formation capabilities of two *Candida albicans* and two *Candida dubliniensis* strains under subMIC concentrations of two antifungal agents. The antifungal agents tested included one fungicidal (amphotericin B) and one fungistatic (fluconazole), corresponding to the most widely used. The quantification of the number of adhered cells and of the biomass present in the biofilm was obtained using two different methods. The total number of cells was determined by violet crystal staining. The tetrazolium salts formation was used to quantify the biofilm activity. The influence of antifungal agents was studied either in initially adhered cells or biofilm formed on acrylic.

5.2 – Materials and Methods

5.2.1 – Media

Cells were grown in Sabouraud dextrose broth (SDB - Merck), that is the most common medium used to grow *Candida* species. Cells were also grown in artificial saliva growth medium. The adhesion assays were performed in ultrapure water. Biofilms were formed in SDB and artificial saliva growth medium.

Sabouraud dextrose medium

The yeast cells were maintained in Sabouraud dextrose agar (SDA) that was prepared according to the manufacturer's instructions (30 g l^{-1}) plus 1.7 % of added agar (Merck). Sabouraud dextrose broth (SDB – Merck) was used as growth liquid medium and prepared using 30 g l^{-1} in water.

Artificial saliva growth medium

In some experiments artificial saliva was used to mimic the *in vivo* oral conditions. This saliva was prepared according to Gal *et al.* (2001) with the following composition in mg l^{-1}: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5 KH_2PO_4, 200.0 Urea, 763.2 $Na_2SO_4.10H_2O$, 178.0 NH_4Cl, 227.8 $CaCl_2.2H_2O$, 630.8 $NaHCO_3$. In order to obtain an artificial saliva growth medium, 2 g of glucose, 2 g of yeast extract and 5 g of peptone were added to the formulation described (Johnson *et al.*, 2000). The pH was adjusted with carbon dioxide to 6.8.

5.2.2 – Antifungal agents

The antifungal agents used were: fluconazole (Pentafarma) and amphotericin B (ICN). Fluconazole was diluted in water until a subinhibitory concentration of 0.25 μg ml^{-1}. In the case of amphotericin B, the first dilution was in DMSO, once it is not water soluble, and the further dilutions were made in water to reach a subinhibitory

concentration of 0.01 μg ml^{-1}. The antifungal solutions were prepared immediately before each experiment.

The subinhibitory concentration of the antifungal agents was determined based in the minimal inhibitory concentrations (MIC) of each agent. The average MIC range presented in Table 5.1 was obtained in a previous work (Cardoso, 2004). These values correspond to the average MIC range for the two *Candida albicans* and the two *Candida dubliniensis* strains assayed. The subinhibitory concentrations (SubMIC) of amphotericin B and fluconazole are also presented in Table 5.1.

Table 5.1 – Average MIC range and subinhibitory concentrations of amphotericin B and fluconazole for *Candida albicans* and *Candida dubliniensis*

	MIC 80 (μg ml^{-1})		SubMIC
	C. albicans	*C. dubliniensis*	(μg ml^{-1})
Amphotericin B	0.006 – 0.02	0.008 – 0.02	0.01
Fluconazole	0.3 – 0.6	0.5 – 0.7	0.25

The selected value of subinhibitory concentrations was the same for both strains once they present similar values of MIC range.

Before adhesion and biofilm formation assays cells were grown in artificial saliva growth medium supplemented with subinhibitory concentrations of the antifungal agents.

5.2.3 – Yeast cells

Two strains of *Candida albicans* and two strains of *Candida dubliniensis* were used in this work. In the case of *Candida albicans* one strain was from American Type Culture Collection, ATCC 32354 (*Candida albicans* B311) and the other was a clinical isolate (*Candida albicans* 12A). In the case of *Candida dubliniensis*, the two strains were obtained from CBS (*Candida dubliniensis* 7987 and *Candida dubliniensis* 7988).

For all the assays, yeast cells were grown for 24 h in SDA at 37 °C. The cells were then inoculated in SDB for 18 h at 37 °C and 150 rpm. After this, 10^8 cell ml^{-1} were diluted 1:10 in artificial saliva growth medium (used as control) and in the same

medium supplemented with subinhibitory concentrations of both antifungal agents. Cells were grown in these media for 24 h at 37 °C and 150 rpm. After, cells were harvested by centrifugation for 10 min at 5000 rpm and 4 °C and washed twice with ultrapure sterile water.

5.2.4 – Acrylic surfaces

The acrylic coupons were prepared as described by Samaranayake and MacFarlane (1980). Briefly, 1.5 g of self-polymerizing acrylic powder was mixed with 1 ml of the monomer in liquid phase and, after mixing, the solution was poured onto a surface covered with aluminium foil. After 45 s another foil was placed on top of the polymerizing mixture. The acrylic sheet, polymerized during 30 min, was cut into 8 × 8 mm^2.

5.2.5 – Adhesion assays

Coupons of acrylic were inserted in a microtiter plate of 24 wells and 2 ml of a cell suspension of 10^7 cells ml^{-1}, prepared with water, were added to each well. After 1 h of incubation (100 rpm, at 37°C) each well was washed twice with ultrapure water, by removing carefully only the liquid above the coupon. Finally all the liquid was removed. These assays were done in triplicate for each strain and the experiment was repeated in two separate occasions.

5.2.6 – Biofilm formation

The biofilm formation was performed using two different approaches. In the first case the biofilm was formed in SDB or artificial saliva growth medium and the enumeration of cells was done after 24 h. In the other assay, biofilms were formed in artificial saliva growth medium with and without fluconazole and amphotericin B. The media were changed each 12 h and the biofilms analysed after 7, 14, 24, 48 and 78 h of formation.

In both cases the biofilm was formed on acrylic coupons (8×8 mm^2) in 24 well plates using a 2 ml yeast cell suspension (10^7 cell ml^{-1}) as innoculum. The experiments were performed in triplicate and repeated twice.

5.2.7 – Cellular quantification

Crystal violet

After either the adhesion or the biofilm formation assays, the coupons were removed from each well and immersed in a new microtiter plate containing 1 ml of methanol in each well. Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. After that, 600 µl of crystal violet were added to each well and incubated for 5 min. The coupons were then gently washed in water and immersed in 1 ml of acetic acid (33 %) to release and dissolve the stain. The absorbance of the obtained solution was read at 570 nm.

Tetrazolium salts

After biofilm formation, the coupons were withdrawn from each well and immersed in a new microtiter plate containing in each well 1 ml of a solution of 100 µg $µl^{-1}$ of XTT and 10 µg $µl^{-1}$ of PMS. The microtiter plate was incubated in the dark for 3 h with agitation (150 rpm). The solution from each well was centrifuged for 3 min at 1000 rpm and the absorbance of the surpernatant was read at 490 nm.

In order to determine if the presence of antifungal agents would influence the formation of formazan salts and the accuracy of this method, several dilutions of *Candida albicans* 12A cells, grown in artificial saliva growth medium (control) and artificial saliva growth medium supplemented with fluconazole or amphotericin B, were incubated with XTT (Figure 5.6).

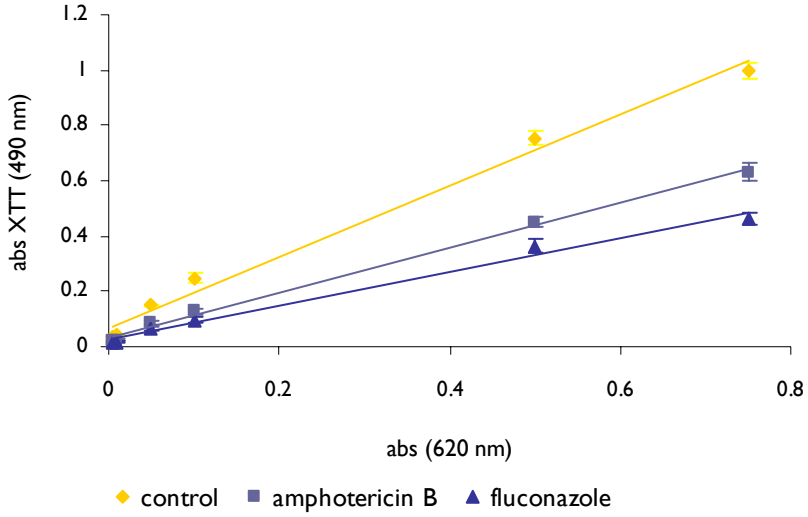


Figure 5.6 – Absorbance (620 nm) of a cell suspension of *Candida albicans* 12A versus the absorbance of the same suspension stained with XTT for cells grown in artificial saliva growth medium (control r^2=0.989) and artificial saliva growth medium supplemented with subMIC concentrations of amphotericin B (r^2=0.996) or fluconazole (r^2=0.989), p=0.000 in all cases.

Observing Figure 5.6, it is possible to note that the curves have different slopes. However, in all cases, the absorbance of XTT solutions presented a good correlation with the cellular concentration given by the absorbance of standard cellular suspensions at 620 nm, corroborated by the significance value (p) that is equal to 0 in all situations assayed.

5.2.8 – Surface properties

Cell lawns were prepared by vacuum filtering a cell suspension of 10^9cells ml^{-1}, through a 3 μm membrane. The membrane was cut in three parts and placed in a Petri dish containing 20 g l^{-1} of agar, and 10 % (v/v) of glycerol for 2 h 30 min. Contact angles were measured by the sessile drop technique using an apparatus model OCA 15 PLUS, DATAPHYSICS.

The measurements were performed, at room temperature, using three different liquids: water, formamide and α-bromonaphthalene. Each assay was performed in triplicate and at least 10 contact angles, per sample, were measured.

The calculation of the free energy and surface tension parameters is presented in chapter 2.

5.2.9 – Statistical analysis

The resulting data were analysed using SPSS (Statistical Package for the Social Sciences). One way ANOVA with Bonferroni test was used for the different comparisons. All tests were performed with a confidence level of 95 %.

5.3 – Results

The effect of subMIC was studied either in adhesion phenomenon and biofilm formation. To complement these studies the surface properties of the cells, grown in subMIC concentrations of the two antifungal agents were determined. As all these studies are independent they will be described in different sub-sections.

5.3.1 – Adhesion studies

The quantification of adhered cells grown in subMIC concentrations of amphotericin B and fluconazole (Figure 5.7) was assessed by crystal violet (CV) staining. In the control (blank experiment) the cells were grown in artificial saliva growth medium without the antifungal agents. The adhesion assays were performed in water in order to avoid other influences of the medium.

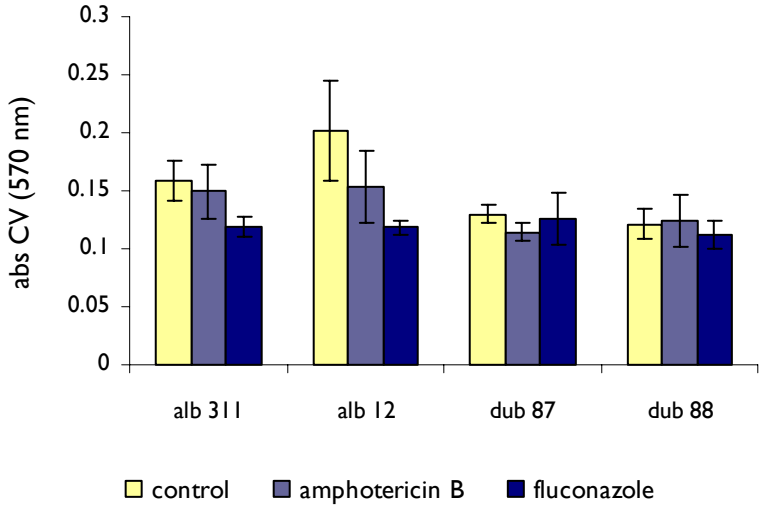


Figure 5.7 – Values of absorbance obtained with CV staining of cells of *Candida albicans* B311 (alb 311), *Candida albicans* 12A (alb 12), *Candida dubliniensis* 7987 (dub 87), *Candida dubliniensis* 7988 (dub 88) , grown in subMIC of amphotericin B and fluconazole, adhered to acrylic.

Figure 5.7 shows that no significant differences were found among *Candida dubliniensis* strains grown in different media. Comparing the adhesion extents of all *Candida* strains grown in the control media, *C. albicans* 12A was able to adhere in a greater number than the other strains.

The growth medium only influenced the adhesion of *Candida albicans* strains as can be seen in Figure 5.7. These strains when grown in subMIC concentrations of fluconazole become less adherent ($p=0.01$). Cells of *Candida albicans* grown in amphotericin B were equally adherent than cells grown in the control media ($p=0.198$).

5.3.2 – Biofilm formation

The assays of biofilm formation included:

- the quantification of biomass formation after 1 day either in SDB or artificial saliva growth medium, using cells grown in subMIC concentrations of antifungal agents or in the control medium;
- the quantification of activity and biomass of biofilms formed over time in subMIC concentrations of amphotericin B and fluconazole.

The first type of assays was performed using CV staining to quantify the biomass of biofilms formed on acrylic and maintained in either SDB (Figure 5.8) or artificial saliva growth medium (Figure 5.9).

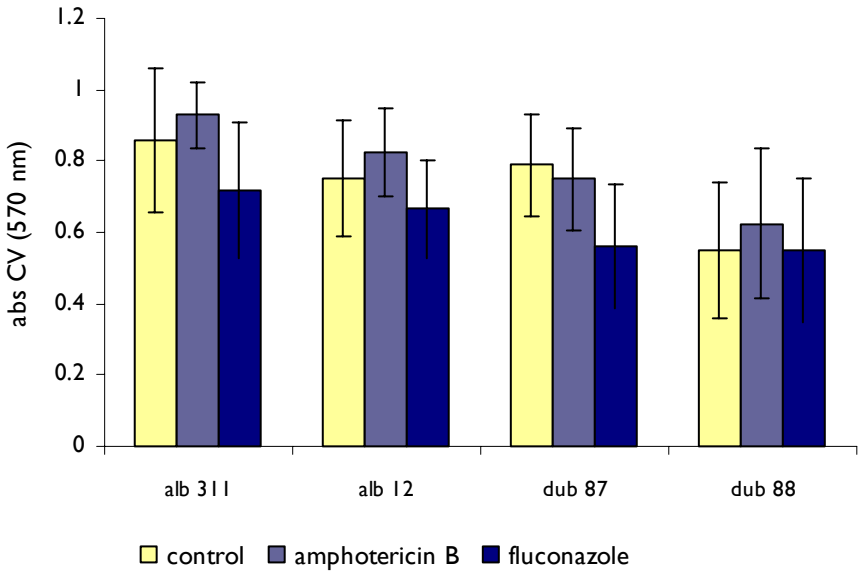


Figure 5.8 – Values of CV staining absorbance of biofilms, formed in SDB, of *Candida albicans* B311 (alb 311), *Candida albicans* 12A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7987 (dub 88) grown in the control and subMIC of amphotericin B (0.01 μg ml^{-1}) and fluconazole (0.25 μg ml^{-1}).

Figure 5.8 shows that all strains formed biofilms with equal biomass, evaluated by CV absorbance, with the exception of *C. dubliniensis* 7988. In the case of this strain some differences in the biofilm biomass were found when compared with the biofilm of the other strains, namely with *Candida albicans* strains in the case of amphotericin B ($p = 0.000$ for *Candida albicans* B311 and $p = 0.017$ for *Candida albicans* 12A)

Figure 5.8 also shows that cells grown in subMIC concentrations of amphotericin B and fluconazole formed the same amounts of biofilm as well as when grown in the control medium, this happens for all the strains.

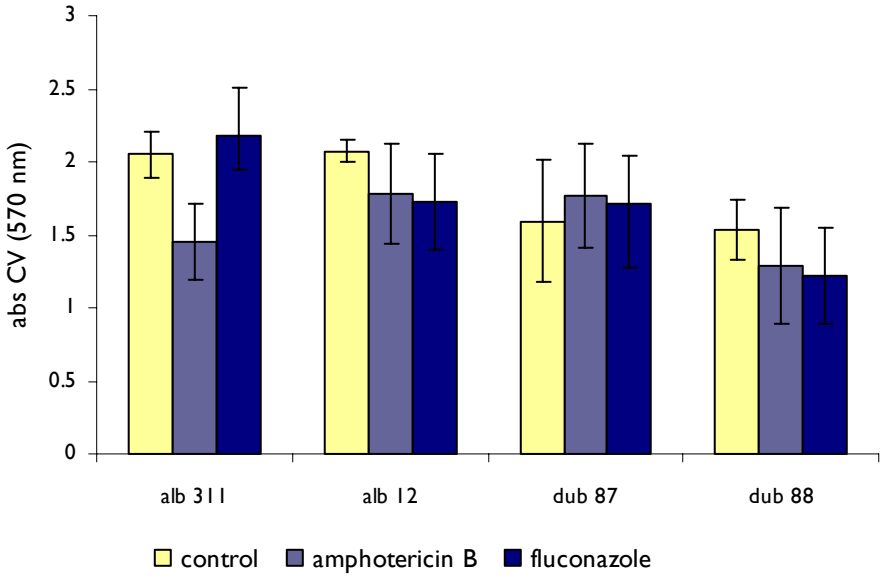


Figure 5.9 – Values of CV staining absorbance of biofilms, formed in artificial saliva growth medium, of *Candida albicans* B311 (alb 311), *Candida albicans* 12A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7987 (dub 88) grown in the control and subMIC of amphotericin B (0.01 μg ml^{-1}) and fluconazole (0.25 μg ml^{-1}).

The biofilms formed in artificial saliva growth medium presented equal amounts of biomass. The exceptions are the biofilms formed by *Candida dubliniensis* 7988. This biofilm biomass presented some statistical differences when compared with biofilms formed by the other strains, namely in the case of fluconazole with p = 0.000 for *C. albicans* B311, p = 0.005 for *C. albicans* 12A and p = 0.006 for *C. dubliniensis* 7987. In the case of artificial saliva growth medium (Figure 5.9) the biofilms formed by all strains grown in subMIC concentrations of fluconazole and amphotericin B presented the same amount of biomass than the ones formed by the same strains when grown in the control medium, with the exception of the strain *C. albicans* B311.
In relation to both biofilm formation medium is it possible to see that the amount of biofilm formed in artificial saliva is higher than when formed in SDB.

The assessment of biofilm formation by cells exposed to subMIC concentrations of the antifungal agents was followed by two different approaches, biomass quantification (CV) and cellular activity (XTT) evaluation (Figures 5.10 and 5.11). Both methods were already used in the study of biofilm formation presented in Chapter 4.

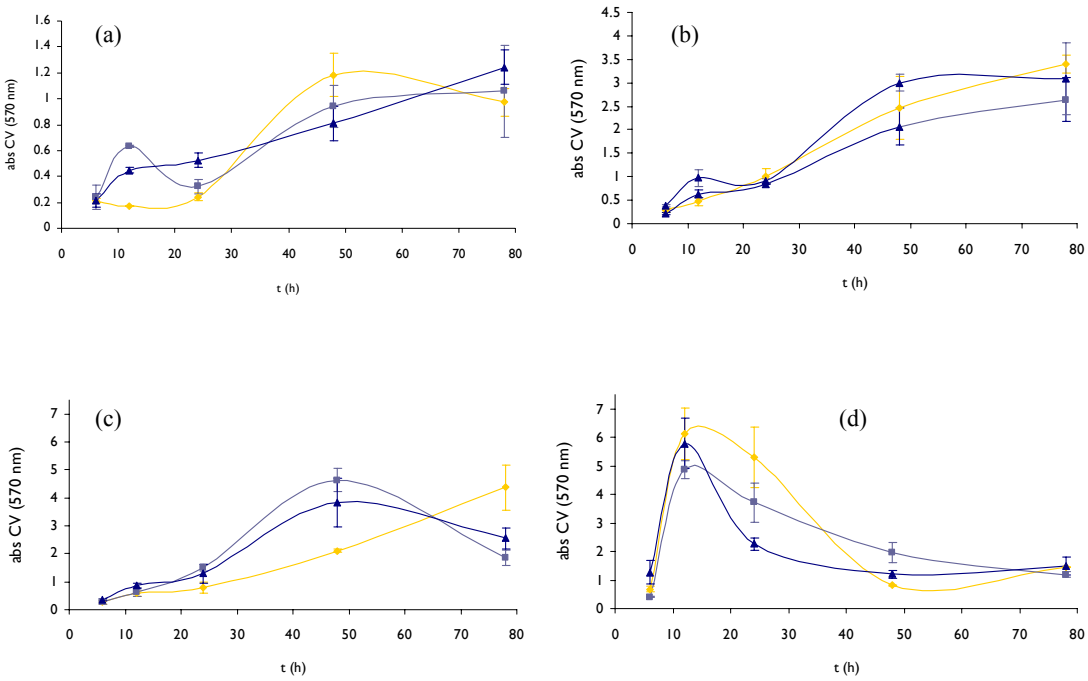


Figure 5.10 – Absorbance obtained from biofilms formed by *Candida albicans* B311 (a), *Candida albicans* 12A (b), *Candida dubliniensis* 7987 (c), *Candida dubliniensis* 7988 (d), stained with CV and grown in control (—) and subMIC concentrations of amphotericin B (—) and fluconazole (—) versus time.

The first point that is notorious from the analysis of Figure 5.10 is that the evolution of *Candida albicans* strains biofilms differs from those of *Candida dubliniensis* strains. The latter form a high amount of biofilm in the early stages (especially *C. dubliniensis* 7988) than the former. In relation to *C. dubliniensis* strains there is a decrease in the amount of biofilms formed, after 48h for *Candida dubliniensis* 7987 and 14 h for *Candida dubliniensis* 7988, whatever the media. The only exception is *Candida dubliniensis* 7987 biofilm formed in the control medium, for which the biofilm increased during the 78 hours of assay. From Figure 5.10 it is possible to observe that biofilms formed by *Candida dubliniensis* 7988 presented the highest levels of biomass while biofilms of *Candida albicans* B311 presented the lowest values of biomass. Actually, the biomass of biofilms formed by Candida albicans B311 was always very small, this could be more evident if Figure 5.10(a) had the same Y axis scale as Figure 5.10 (b). However, if such scale was used it was not possible to distinguish the effect of subMIC concentrations of the antifungal agents.

Considering *C. albicans* 12A, the biofilm formation profile in amphotericin B and in the control presented a plateau after 48 h, while the biofilm formation profile in fluconazole still increased after that time. In the case of *C. albicans* B311 the biofilm

formed in the presence of fluconazole attained a plateau after 48h. While the biofilm formation profile in the control and amphotericin B still increased until 78h.

In order to interpret the results (Figure 5.10) a statistical analysis (one-way ANOVA) was used to compare the biofilms formed in the different media (Table 5.2) and the biofilms formed by the different strains (Table 5.3). The comparisons were performed for 24, 48 and 78 h, once these periods of time represent the major differences.

Table 5.2 – Significance value (p) obtained from the comparison of the different media used for *Candida albicans* B311 (alb 311), *Candida albicans* 12A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88) biofilm formation after 24, 48 and 78 h

		alb 311		alb 12		dub 87		dub 88	
		amph	fluc	amph	fluc	amph	fluc	amph	fluc
24h	cont	0.012	0.000	0.014	0.157	0.000	0.000	0.001	0.000
	amph	--	0.000	--	0.907	--	0.227	--	0.005
48h	cont	1.000	1.000	0.366	0.009	0.000	0.000	0.000	0.002
	amph	--	0.397	--	0.000	--	0.030	--	0.000
78h	cont	0.158	0.054	0.090	1.000	0.000	0.000	0.351	1.000
	amph	--	1.000	--	0.335	--	0.045	--	0.095

Observing Table 5.2 the influence of fluconazole on *C. dubliniensis* biofilms is more notorious, than in *C. albicans* biofilms ($p>0.05$ in the case of 48 and 72h *Candida albicans* B311 biofilms and in the case of 78h *Candida albicans* 12A biofilms).

From Figure 5.10 and Table 5.2 it is also possible to observe that for 24h and 48h there are some differences ($p<0.05$) in the biofilm total biomass formed in subMIC concentrations of the antifungal agents (except for *Candida albicans* B311 biofilms formed at 48h) while for 72h in most cases no differences were encountered ($p>0.05$), with the exception of *Candida dubliniensis* 7987 biofilms.

Table 5.3 – significance value (p) obtained from the comparison of the different biofilms of *Candida albicans* B311 (alb B311), *Candida albicans* 12A (alb 12), *Candida dubliniensis* 7987 (dub 87) and *Candida dubliniensis* 7988 (dub 88) formed in artificial saliva growth medium in the absence or presence of antifungal agents, after 24, 48 and 78 h

		control			Amphotericin B			Fluconazole		
		alb 12	dub 87	dub 88	alb 12	Dub 87	dub 88	alb 12	dub 87	dub 88
24h	alb B311	0.042	0.280	0.000	0.003	0.000	0.000	0.005	0.000	0.000
	alb12	--	1.000	0.000	--	0.001	0.000	--	0.005	0.000
	dub 87	--	--	0.000	--	--	0.000	--	--	0.000
48h	alb B311	0.000	0.000	0.081	0.000	0.000	0.000	0.000	0.000	1.000
	alb12	--	0.478	0.000	--	0.000	1.000	--	0.067	0.000
	dub 87	--	--	0.000	--	--	0.000	--	--	0.000
78h	alb B311	0.000	0.000	0.894	0.000	0.002	1.000	0.000	0.000	1.000
	alb12	--	0.007	0.000	--	0.000	0.000	--	0.067	0.000
	dub 87	--	--	0.000	--	--	0.003	--	--	0.000

As regards the comparison between the biofilms formed by *Candida* strains (Figure 5.10 and Table 5.3) it can be stressed that the different strains present different behaviour, concerning biofilm formation in the presence of antifungal agents. This corroborates the previous analysis of Figure 5.10

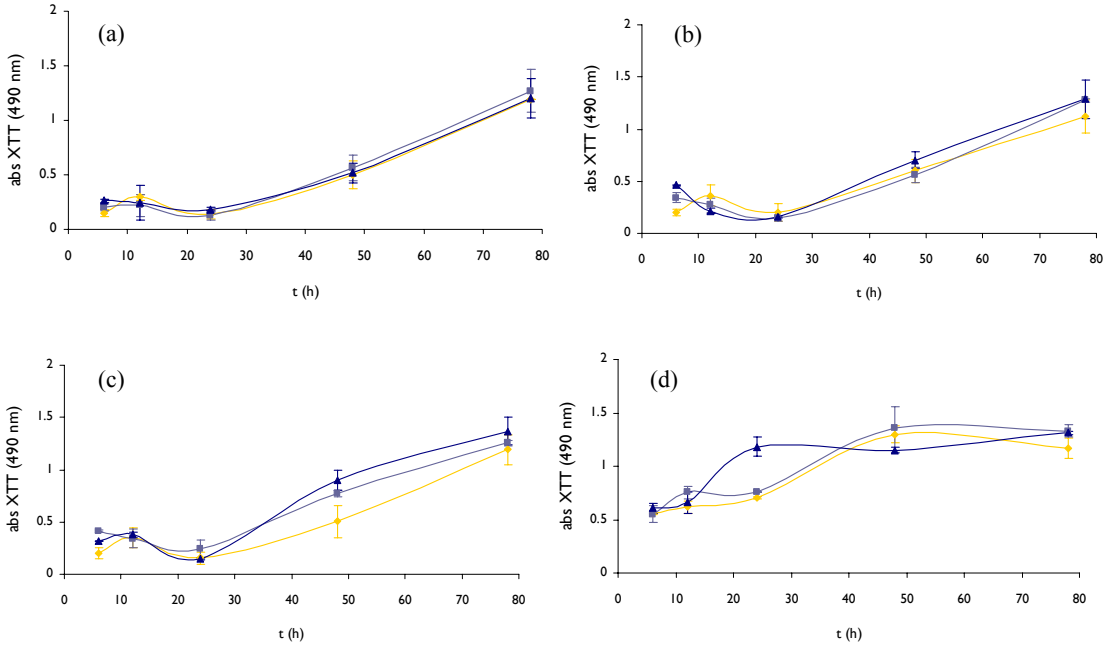


Figure 5.11 – Absorbance obtained from biofilms formed by *Candida albicans* B311 (a), *Candida albicans* 12A (b), *Candida dubliniensis* 7987 (c), *Candida dubliniensis* 7988 (d), stained with XTT and grown in control (—) and in subMIC concentrations of amphotericin B (—) and fluconazole (—)versus time.

Concerning the biofilm activity (Figure 5.11), biofilms of *Candida albicans* B311, *Candida albicans* 12A and *Candida dubliniensis* 7987 presented similar activities in media with or without subMIC concentrations of amphotericin B and fluconazole. Contrary to the other strains, biofilms of *Candida dubliniensis* 7988 displayed (Figure 5.11d) a plateau of activity after 48h when formed in the control media and in the presence of amphotericin B and after 24h in the presence of fluconazole.

The differences on the biofilm activities of the different species, assessed by XTT, are not so notorious, but still detectable. In the case of both strains of *Candida albicans* the biofilm formation profiles in the three situations are very similar, while for the biofilms of the other species there are more oscillations, being the differences more significant in the case of biofilms of *C. dubliniensis*7988. This biofilm already presented the major difference when the biomass was quantified. The amount of biofilm produced by *Candida dubliniensis* strains is higher than the amount of *Candida albicans* strains biofilm (Figure 5.10). Accordingly, in all cases it is possible to see (Figure 5.11) that the activity is low in the earlier stages and increases with the time.

Therefore, *Candida dubliniensis* biofilms activities are also higher than that of *Candida albicans* biofilm activities.

5.3.3 – Surface properties

In order to explain the adhesion phenomenon and biofilm formation, cell surface properties of cells grown in artificial saliva growth medium without and with subMIC concentrations of antifungal agents were determined. The determination of cell surface properties involved the measurement of contact angles (Table 5.4) and the subsequent calculation of the surface tension parameters and free energy (Table 5.5).

Table 5.4 – Values of contact angles measured with water (θ_w), formamide (θ_f) and α-bromonaphatelene (θ_b) on cells lawns of *C. albicans* B311, *C. albicans* 12A, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 grown in artificial saliva growth medium (Control) and artificial saliva growth medium supplemented with subMIC concentrations of amphotericin B and fluconazole

Medium	Cells	θ_w (°) (±SD[a])	θ_f (°) (±SD[a])	θ_b (°) (±SD[a])
Control	*C. albicans* B311	15 ± 1	18 ± 1	55 ± 3
	C. albicans 12A	18 ± 2	15 ± 3	48 ± 5
	C. dubliniensis 7987	14 ± 1	13 ± 0	41 ± 0
	C. dubliniensis 7988	17 ± 1	22 ± 0	42 ± 4
Amphotericin B	*C. albicans* B311	13 ± 2	16 ± 0	54 ± 8
	C. albicans 12A	18 ± 2	19 ± 2	46 ± 2
	C. dubliniensis 7987	15 ± 0	15 ± 0	40 ± 0
	C. dubliniensis 7988	16 ± 1	22 ± 0	49 ± 3
Fluconazole	*C. albicans* B311	16 ± 2	18 ± 1	43 ± 2
	C. albicans 12A	16 ± 2	12 ± 1	50 ± 7
	C. dubliniensis 7987	15 ± 0	14 ± 0	41 ± 1
	C. dubliniensis 7988	19 ± 3	20 ± 1	45 ± 2

[a]SD, Standard Deviation

From Table 5.4 it can be concluded that all the strains have similar contact angles, formed with the three liquids. Considering the water contact angle, it is lower than 50° (van Oss and Giese, 1995) in all cases, which means that all the strains should present a hydrophilic character.

The determination of the surface tension components and the free energy of interaction, ΔG_{sws} (between two cells of the same strain immersed in water – degree of hydrophobicity) was done according to Van Oss and Giese (1995) using the equations presented in Chapter 2.

Table 5.5 – Values of the components of surface tension (γ^+, γ^-, γ^{LW}) and degree of hydrophobicity (ΔG_{sws}) of cells (*C. albicans* 12A, *C. albicans* 46B, *C. dubliniensis* 7987 and *C. dubliniensis* 7988) conditioned with water or artificial saliva and the average zeta potential of the yeast cells measured in water

Medium	Cells	γ^+ (mJ m^{-2}) (±SD[a])	γ^- (mJ m^{-2}) (±SD[a])	γ^{LW} (mJ m^{-2}) (±SD[a])	ΔG_{sws} (mJ m^{-2}) (±SD[a])
Control	*C. albicans* B311	4 ± 0	53 ± 2	28 ± 1	26 ± 1
	C. albicans 12A	3 ± 1	50 ± 0	31 ± 2	25 ± 2
	C. dubliniensis 7987	2 ± 0	53 ± 1	34 ± 2	28 ± 1
	C. dubliniensis 7988	2 ± 0	54 ± 0	34 ± 2	32 ± 0
Amphotericin B	*C. albicans* B311	5 ± 2	54 ± 0	27 ± 5	26 ± 4
	C. albicans 12A	3 ± 0	52 ± 1	32 ± 1	28 ± 1
	C. dubliniensis 7987	2 ± 0	53 ± 0	35 ± 0	29 ± 0
	C. dubliniensis 7988	3 ± 0	55 ± 0	31 ± 1	30 ± 0
Fluconazole	*C. albicans* B311	2 ± 0	53 ± 1	33 ± 1	30 ± 0
	C. albicans 12A	4 ± 1	51 ± 1	30 ± 5	24 ± 1
	C. dubliniensis 7987	2 ± 0	52 ± 0	34 ± 0	28 ± 0
	C. dubliniensis 7988	2 ± 0	52 ± 2	32 ± 1	28 ± 3

[a]SD, Standard Deviation

The values of Table 5.5 confirm the hydrophilic character of all the strains once ΔG_{sws} is always positive, this confirms the results obtained with the water contact angle (Table 5.5). Corroborating the similarity of the values of the contact angles all the values of surface tension parameters and degree of hydrophobicity are similar for all conditions as well ($p>0.05$).

5.4 – Discussion

The effect of subMIC concentrations of antifungal agents in the adhesion of *Candida* strains was assessed in this study by means of crystal violet staining. In the previous adhesion study (Chapter 2) direct cell enumeration was used to quantify the extent of adhesion. Although this method is more accurate than indirect techniques it is more time consuming and can not be used to assess biofilm biomass. Therefore, in this part of the work CV staining was used to quantify both adhered cells and biofilm biomass.

The results obtained showed that when cells of the different strains are exposed to subMIC concentrations of either the fungicidal amphotericin B or the fungistatic

fluconazole their extent of adhesion to acrylic is similar, except for Candida albicans strains grown in subMIC concentrations of fluconazole (Figure 5.7). The results presented in Chapter 2 also demonstrated that the different species and strains presented the same extents of adhesion, although in this case the growth medium was different. This similar phenotypic behaviour can be explained by the analogous cell surface properties. It became clear from the previous study and corroborated by other authors (Doyle, 2000; Panagoda *et al.*, 1998; Panagoda *et al.*, 2001) that cell surface properties influence the adhesion to inert surfaces. Accordingly, as the growth media supplemented with subMIC concentrations of the antifungal agents did not modify the surface tensions and hydrophobicity of all *Candida*l species and strains, subMIC concentrations of antifungal agents had no effect on yeast cell adhesion.

There are only few studies reporting the influence of subinhibitory concentrations of antifungal agents in yeast cell surface properties. According to Hazen *et al.* (2000), *Candida albicans* cell surface hydrophobicity degree of treated cells was not significantly different from the control. The results obtained in the present work lead to the same conclusion, since all the strains grown in the presence or absence of antifungal agents presented a similar degree of hydrophilicity.

The ability of both species and strains, when grown in subMIC concentrations of the antifungal agents, to form biofilms was the same as when grown in the control medium, this was assessed by studying the 24 h biofilms (Figures 5.8 and 5.9). Artificial saliva growth medium promoted the formation of a higher amount of biofilm biomass, when compared with SDB. The results reported in the previous chapter also pointed out that artificial saliva enhances biofilm formation. One of the reasons for this fact is the high amount of hyphae formation in this medium, which probably contributes to a higher biofilm biomass.

SubMIC concentrations of antifungal agents did not influence biofilm formation profiles of all strains. This was assessed by measuring biofilm biomass and activity during different periods of biofilm formation in the presence of subMIC concentrations of amphotericin B and fluconazole (Figure 5.10). The biofilm formation of *Candida albicans* grown in subinhibitory concentrations of voriconazole, nystatin and chlorhexidine using the dry weight and the XTT methods was studied by Kuhn *et al.* (2002a). These

authors concluded that while there were differences in the values of dry weight when cells grew in antifungal agents, considering the results given by XTT there were no statistical differences. Ramage *et al.* (2002) also studied the influence of subinhibitory concentrations of fluconazole and amphotericin B in *Candida* biofilms and drew the conclusion that while fluconazole had no effect on the biofilms, amphotericin B decreases their activity but for concentrations that are high and above the therapeutic margin. The present study leads to the same conclusions, with subMIC concentrations of antifungal agents having no effect on biofilm activity, probably because the concentrations used were below the concentration for which cells are really susceptible.

The results present in this chapter shows differences between the biofilm evolution profiles in the presence of subMIC concentrations of the antifungal agents among the different strains and species. According to the results reported in the previous chapter, biofilm formation profile is also strain and species dependent, although biofilm formation medium was different. The differences encountered were notorious when considering biofilm biomass. *Candida albicans* strains present a common biofilm formation profile (Figures 5.10a and 5.10b), with the biofilm biomass increasing along the time, while *Candida dubliniensis* strains (Figures 5.10c and 5.10d) form the major amount of biofilm in the earlier stages. Biofilms of *Candida dubliniensis* 7988 display initially a great amount of biomass accumulation followed by a decrease that can be explained by the detachment of the excessive mass of biofilm.

Analysing the biofilm activity profiles (Figure 5.11), it can be noticed that the activity is higher for the higher levels of biomass, which explains the activity profile of *Candida dubliniensis* 7988. Biofilms formed by both strains of *Candida dubliniensis* present higher levels of activity corresponding to stages of high biomass accumulation and those levels of activity are still maintained even when the biomass decreases. A possible explanation is that the medium where biofilms were formed was changed every 24 h, allowing the cells to remain active.

Corroborating the results presented in the previous chapter, concerning the biofilm formation assessed by CV and XTT, the biofilms formed in subMIC concentrations of fluconazole and amphotericin B are strain and species dependent.

Chapter 6 - CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The main goal of the subject of this dissertation was the comparison between a recently discovered *Candida* species (*Candida dubliniensis*) and the well known *Candida albicans*, in terms of their ability to adhere and form biofilms in the oral cavity.

The main conclusions drawn from the results obtained are that adhesion to self-polymerizing acrylic (used in prosthetic devices) and HAP (simulating tooth enamel) is not species dependent, while adhesion to epithelium and biofilm formation is species and strain dependent. The influence of subinhibitory concentrations of amphotericin B and fluconazole was not notorious either in the adhesion phenomenon or in biofilm formation.

As more particular conclusions are concerned, the following should be highlighted:

Considering cell surface properties (surface tension parameters, degree of hydrophobicity), there were no significative differences among the four strains assayed, neither when these properties were measured with cells conditioned by different solutions (water, artificial saliva solution, saline solution or after growth in subMIC concentrations of fluconazole and amphotericin B).

The number of adhered cells of both species and both strains, to inert surfaces (self-polymerizing acrylic and HAP), were statistically similar.
For all strains and both species, the extent of adhesion to inert surfaces in the presence of artificial saliva increased on acrylic and had no alteration on HAP, when compared with water as the adhesion medium.
When water was used as adhesion medium, the amount of adhered cells to HAP was higher than the amount adhered to acrylic. However, when artificial saliva was used this difference was not found.

Artificial saliva induced some differences in the extent of adhesion to epithelial HeLa cells among the different strains. The number of attached *Candida albicans* to epithelial cells in the presence of artificial saliva was higher than when in saline solution. The opposite was obtained for *Candida dubliniensis* 7988.

The biofilm profile versus time was characteristic of each strain, both in terms of biomass accumulation and activity.

The biofilm biomass evolution with time was different between biofilms formed in SDB and artificial saliva growth medium. Biofilms formed in artificial saliva growth medium displayed higher activity than when formed in SDB.

Pseudohyphae were present in all the biofilms formed but there was no evidence of a direct effect on the amount of biomass accumulated.

The effect of sub inhibitory concentrations of the antifungal agents on the growth of yeast cells and consequent extent of adhesion and biofilm formation was not notorious.

There were some differences in biomass accumulation among the biofilms of the strains assayed when they where formed in subMIC concentrations of antifungal agents.

The first consideration about future work is the suggestion to use clinical strains of each species, especially of *Candida dubliniensis*. The clinical strains should be obtained from patients with and without oral candidiasis or other diseases (e.g. HIV) and using or not prosthetic devices. A larger array of samples would help confirming the results and understanding strain variations.

As the oral cavity contains yeasts and bacteria, studies involving their interactions should also be considered.

Other materials then self-polymerized acrylic and HAP should be assayed, especially:

- soft linning materials (plasticized acrylics and silicone elastomers)
- methalic alloys (Nickel–Chromium-Molybdenum, Nickel–Chromium–Beryllium)

It should be interesting to study the effect of the combination of the two antifungal agents studied and also new agents, likewise Caspofungin.

The determination of both species factors of virulence as secreted aspartyl proteases (SAPs) and the presence of adhesins is also a research challenge.

Another point to consider is the use of cell lines related with the oral cavity or primary explants, in order to mimic more close *in vivo* conditions.

REFERENCES

Adam B., Baillie G.S., and Douglas J. (2002) Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *Journal of Medical Microbiology*. **51:** 344-349.

Aguedo, M., Waché Y., Mazonyer V., Sequeira-Le Grand A., and Belin J. (2003) Increased Electron donor and electron acceptor characters enhance the adhesion between oil droplets and cells of *Yarrowia lipolytica* as evaluated by new cytometric assay. *Journal of Agricultural and Food Chemistry*. **51:** 3007-3011.

Altman F.P. (1976) Tetrazolium salts and formazans. *Prog Histochemistry Cytochemistry*. **9:** 1-56.

Arthington-Skaggs B.A., Motley M., Warnock D.W., and Morrison C.J. (2000) Comparative Evaluation of PASCO and National Committee for Clinical Laboratory Standards M27-A Broth Microdilution Methods for Antifungal Drug Susceptibility Testing of Yeasts. *Journal of Clinical Microbiology*. **38:** 2254-2260.

Arzeni D., Barchiesi F., Compagnucci P., Cellini A., Simonetti O., Offidani A.M., and Scalise G. (1998) In vitro activity of terbinafine against clinical isolates of dermatophytes. *Medical Mycology*. **36:** 235-237.

Bachmann S.P., Walle K.V., Ramage G., Patterson T.F., Wickes B.L., Graybill J.R., and López-Ribot J.L. (2002) In vitro activity of Caspofungin against *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy*. **46:** 3591-3596.

Bailey A., Wadsworth E., and Calderone R.A. (1995) Adherence of *Candida albicans* to human buccal epithelial cells: Host Induced Protein Synthesis and Signaling Events. *Infection and Immunity*. **63:** 569-572.

Baillie G.S. and Douglas J. (2000) Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *Journal of Antimicrobial Chemotherapy*. **46:** 397-403.

Baillie G.S. and Douglas L.J. (1999) Role of dimorphism in the development of *Candida albicans* biofilms. *Journal of Medical Microbiology*. **48:** 671-679.

Beighton D., Ludford R., Clark D.T., Brailsford S.R., Pankhurst C.L., Tinsley G.F., Fiske J., Lewis D., Daly B., Khalifa N., Marren V., and Lynch E. (1995) Use of CHROMagar Candida medium for isolation of yeasts from dental samples. *Journal of Clinical Microbiology*. **33:** 3025-3027.

Berg F.T. (1846) Om Torsk Hos Barn. *L.J.Hjerta, Stockholm, Sweeden*.

Berkhout C.M. (1923) De schimmelgeschlachten Monilia, Oidium, oospora, en Torula. University of Utrech, The Netherlands

Berry E.D. and Siragusa G.R.. (1997) Hydroxyapatite adherence as a means to concentrate bacteria. *Applied and Environmental Microbiology*. **63:** 4069-4074.

Bolard J. (1986) How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochemistry Biophysics*. **864:** 257-304.

Bolard J. (1991) Mechanism of action of an anti-Candida drug: amphotericin B and its derivatives. *In*:Prasad R. (Eds).*Candida albicans,* Springer-Verlag, Berlin, pp 214-238.

Bollen C.M., Papaioanno W., Eldere J.V., Schepers E., Quirynen M., and van Steenberghe D. (1996) The influence of abutment surface roughness on plaque accumulation and peri-implant mucositis. *Clinical Oral Implants Research*. **7:** 201-211.

Bollen C.M.L., Lambrechts P., and Quirynen M. (1997) Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: A review of the literature. *Dentistry Materials*. **13:** 258-269.

Boonarte C.J. and Rouxhet P.G. (2000) Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. *Applied and Environmental Microbiology*. **66:** 2548-2554.

Bouali A., Robert R., Tronchin G., and Senet J.M. (1986) Binding of human fibrinogen to *Candida albicans* in vitro: a preliminary study. *Journal of Medical and Veterinary Mycology*. **24:** 345-348.

Brawner D.L., Anderson G.L., and Yuen K.Y. (1992) Serotype prevalence of *Candida albicans* from blood cultures isolates. *Journal of Clinical Microbiology*. **30:** 149-153.

Brown A.J.P. (2002a) Morphogenic signaling pathways in Candida albicans. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D. C., pp 95-106.

Brown A.J.P. (2002b) Expression of growth form-specific factors during morphogenesis in Candida albicans. *In*:Calderone R.A. (Eds).*Candida and Candidisis,* ASM Press, Washington D.C., pp 87-93.

Budtz-Jorgensen E. (1972) Denture stomatitis. V. *Candida* agglutinins in human sera. *Acta Odontologica Scandinavica*. **30:** 313-325.

Busscher H.J. and Evans L.V. (1000) Oral biofilms and plaque control,Harwood academic publishers (Eds),

Busscher H.J., Geertsema-Doornbusch G.I., and van der Mei H.C. (1997) Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *Journal of Biomedical Materails Research*. **34:** 201-210.

Calderone R., Suzuki S., and Cannon R.D. (2000) *Candida albicans*: adherence, signaling and virulence. *Medical Mycology*. **38:** 125-137.

Calderone R.A. (2002a) Taxonomy and biology of *Candida*. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D. C., pp 15-27.

Calderone R.A. (2002c) *Candida* and *Candidiasis*, ASM Press (Eds), Washington D.C.

Calderone R.A. (2002b) Introduction and historical prespectives. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D.C., pp 3-13.

Calderone R.A. and Braun P.C. (1991) Adherence and receptor relationships of *Candida albicans*. *Microbiological Reviews*. **55:** 1-20.

Calderone R.A. and Fonzi W.A. (2001) Virulence factors of *Candida albicans*. *Trends in Microbiology*. **9:** 327-335.

Calderone R.A. and Gow N.A.R. (2002) Host recognition by *Candida* species. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D. C., pp 67-86.

Campbell C.K., Davey K.G., Holmes A.D., Szekely A., and Warnock D.W. (1998) Comparison of the API Candida System with the AUXACOLOR system for lientification of common yeast pathogens. *Journal of Clinical Microbiology*. **37:** 821-823.

Cannon R.D. and Chaffin W.L (1999) Oral colonization by *Candida albicans*. *Critical Reviews in Oral Biology and Medicine*. **10:** 359-383.

Cannon R.D., Nand A.K., and Jenkinson H.F. (1995) Adherence of *Candida albicans* to human salivary components adsorbed to hydroxiapatite. *Microbiology*. **141:** 213-219.

Cardoso B.C. (2004) Efeito de antifúngicos em suspensões e biofilmes de *Candida albicans* e *Candida dubliniensis*. Master thesis, Universidade do Minho

Chaffin W.L, López-Ribot J.L., Casanova M, Gozalbo D., and Martinez J.P. (1998) Cell wall and secreted proteins of *Candida albicans*: identification, function and expression. *Microbiology and Molecular Biology Reviews*. **62:** 130-180.

Chandra J., Kuhn D.M, Mukherjee P.K., Hoyer L.L., McCormick T., and Ghannoum M.A. (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. *Journal of Bacteriology*. **183:** 5385-5394.

Chauhan N., Li D., Singh P., Calderone R., and Kruppa M. (2002) The cell wall of *Candida* spp. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D. C., pp 159-175.

Christensen G.D., Simpson W.A., Younger J.J., Baddour L.M., Barrett F.F., Melton D.M., and Beachley E.H. (1985) Adherence of coagulase negative *staphylococci* to plastic tissue culture plates: A quantitative method for the adherence of *staphylococci* to medical devices. *Journal of Clinical Microbiology*. **22:** 996-1006.

Clark W.B., Bammann L.L., and Gibbons R.J. (1978) Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. *Infection and Immunity*. **19:** 846-853.

Coleman D.C., Sullivan D.J., Haynes K., Henman M., Shanley D., Bennett D.E., Moran G.P., McCreary C., O'Neill L., and Harrington B.J. (1997) Molecular and

phenotypic analysis of *Candida dubliniensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients. *Oral diseases*. **3:** 96-101.

Costa L.J., Birman E.G., Alves S.H., and Cury A.E. (1999) Antifungal susceptibility of *Candida albicans* isolated from oral mucosa of patients with cancer. *Revista de Odontologia Universidade de São Paulo*. **13:** 219-223.

Costerton J.W., Marrie T.J., and Cheng K.J. (1985) Phenomenum of bacterial adhesion. *In*:Savage D.C. and Fletcher M. (Eds).*Bacterial adhesion,* Plenum Press, New York, pp 3-43.

Costerton J.W., Stewart P.S., and Greenberg E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science*. **284:** 1318-1322.

Cotter G. and Kavanagh K (2000) Adherence mechanisms of *Candida albicans*. *British Journal of Biomedical Science*. **57:** 241-249.

Cowan M.M., Taylor K.G., and Doyle R.J. (1987) Energetics of the initial phase of adhesion of *Streptococcus sanguis* to hydroxylapatite. *The Journal of Bacteriology*. **169:** 2995-3000.

Crist A.E., Dietz JR.T.J., and Kampschroer K. (1996) Comparison of the Murex *C. albicans,* albicans-sure and BactiCard Candida test kits with the germ tubes test for presumptive identification of *Candida albicans*. *Journal of Clinical Microbiology*. **34:** 2616-2618.

Davenport J.C. (1970) The oral distribution of *Candida* in denture stomatitis. *British Dental Journal*. **129:** 151-156.

Davenport J.C. (1972) The denture surface. *British Dental Journal*. **133:** 101-105.

Donlan R.M. (2002) Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*. **8:** 1-9.

Donlan R.M. (2001) Biofilms and device-associated infections. *Emerging Infectious Diseases*. **7:** 277-281.

Donlan, R. M. and Costerton, J. W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*. **15:** 167-193.

Dorocka-Bobkowska B., Konopka K., and Duzgunes N. (2003) Influence of antifungal polyenes on the adhesion of *Candida albicans* and *Candida glabarata* to human epithelial cells in vitro. *Archives of Oral Biology*. **48:** 805-815.

Douglas J. (1987) Adhesion of *Candida* species to epithelial surfaces. *Critical Reviews in Microbiology*. **15:** 27-43.

Douglas J. (2003) *Candida* biofilms and their role in infection. *Trends in Microbiology*. **11:** 30-36.

Douglas L.J. (2002) Medical importantce of biofilms in *Candida* infections. *Revista Iberoamicana de Micologia*. **19:** 139-143.

Doyle A. (1991) Strategies in experimental microbial adhesion research. *In*:Mozes N., Handley P.S., Busscher H.J., and Rouxhet P.G. (Eds).*Microbial cell surface analysis. Structural and physicochemical methods,* VCH Publishers, New York, pp 293-316.

Doyle A. and Griffiths J.B. (2000) Cell and tissue culture for medical research, John Wiley & Sons Ltd (Eds), West Sussex

Doyle RJ (2000) Contribution of the hydrophobic effect to microbial infection. *Microbes and infection*. **2:** 391-400.

Dufrêne Y.F., van der Wai A., Norde W., and Rouxhet P.G. (1997) X-ray Photoelectron spectroscopy analysis of whole cells and isolated cell walls of gram-positive bacteria: comparison with biochemical analysis. *Journal of Bacteriology*. **179:** 1023-1028.

Edgerton M., Scannapieco F.A., Reddy M.S., and Levine M.J (1993) Human Submandibular-Sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. *Infection and Immunity*. **61:** 2644-2652.

Egusa H., Ellepola A.N.B., Nikawa H., Hamada T., and Samaranayke L.P. (2000) Exposure to subtherapeutic concentrations of polyene antifungals suppresses the adherence of *Candida* species to denture acrylic. *Chemotherapy*. **46:** 267-274.

Elie C.M., Lott T.J., Reiss E., and Morrison C.J. (1998) Rapid identification of *Candida* species with species-specific DNA probes. *Journal of Clinical Microbiology*. **36:** 3260-3265.

Ellepola A.N.B. and Samaranayke L.P. (1998) Adhesion of oral *Candida albicans* isolates to denture acrylic following limited exposure to antifungal agents. *Archives of Oral Biology*. **43:** 999-1007.

Ellewood D.C., Melling J., and Rutter P. (1979) Adhesion of microorganisms to surfaces, Academic Press (Eds), London

Flahaut M., Sanglard D., Monod M., Bille J., and Rossier M. (1998) Rapid Detection of *Candida albicans* in Clinical Samples by DNA Amplification of Common Regions from C. albicans-Secreted Aspartic Proteinase Genes. *Journal of Clinical Microbiology*. **36:** 395-401.

Freed L.E. and Vunjak-Novakovi G. (1995) Cultivation of cell-polymer constructs in simulated microgravity. *Biotechnology and Bioengeneering*. **46:** 306-313.

Freed L.E. and Vunjak-Novakovi G. (2000) Tissue engineering bioreactors. *In*: Lanza R.P., Langer R., and Vacanti J. (Eds).*Principles of tissue engineering,* Academic Press, 143-156.

Fukuzawa Y. and Kagaya K. (1997) Molecular adhesion of *Candida albicans*. *J Med Vet Mycol*. **35:** 99-

Gal J., Fovet Y., and Adib-Yadzi M. (2001) About a synthetic saliva for in vitro studies. *Talanta*. **53:** 1103-1115.

Gale C.A., Bendel C.M., McClellan M., Becker J.M., Berman J., and Hostetter M.K. (1998) Linkage of adhesion, filamentous grwoth and virulence in *Candida albicans* to a single gene, INTI. *Science*. **279:** 1355-1358.

Georgopapadakou N.H. and Walter J.D. (1996) Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrobial Agents and Chemotherapy*. **40:** 279-291.

Ghannoum M.A. and Abu-Elteen K. (1991) Adherence of *Candida albicans*: Influencing Factors and Mechanism(s). *In*:Prasad R. (Eds).*Candida albicans,* Springer-Verlag, Berlin, pp 145-163.

Ghannoum M.A. and Radwan S.S. (1990) Candida adherence to epithelial cells, CRC Press (Eds), kuweit

Gilfillan G.D., Sullivan D.J., Haynes K., Parkinson T., Coleman D.C., and Gow N.A.R. (1998) *Candida dubliniensis*: phylogeny and putative virulence factors. *Microbiology*. **144:** 829-838.

Gruby M. (1842) Recherches anatomiques sur ue plant cryptogame qui constitute le vrai muguet des enfants. *Comptes Rendus de l'Academie de Sciences*. **14:** 634-636.

Gustafson K.S., Vercelloti G.M., and Hostetter M.K. (1991) Molecular mimicry in *Candida albicans*. Role of an integrin analogue in adhesion of the yeast to human endothelium. *Journal of Clinical Investigation*. **87:** 1896-1902.

Hawser S.P., Baillie G.S., and Douglas J. (1997) Production of extracelular matrix by *Candida albicans* biofilms. *Journal of Medical Microbiology*. **47:** 253-256.

Hawser S.P. and Douglas L.J. (1994) Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infection and Immunity*. **62:** 915-921.

Hawser S.P., Norris H., Jessup C.J., and Ghannoum M.A. (1998) Comparison 2,3-bis(2-methoxy-4-nitro-5-sulfenyl)-5-[(Phenylamino) carbonyl]-2H-tetrazolium-hydroxide (XTT)ncolorimetric method with the standardized national committee for clinical laboratory standards method of testing clinical yeast isolates for susceptibility to antifungal agents. *Journal of Clinical Microbiology*. **36:** 1450-1452.

Hazen K.C. (1995) New and emerging yeast pathogens. *Clinical Microbiology Reviews*. **8:** 462-478.

Hazen K.C. and Hazen B.W. (1987) A polysterene microsphere assay for detecting surface hidrophobicity variations within *Candida albicans* populations. *Journal of Microbiological Methods*. **6:** 289-299.

Hazen K.C. and Hazen B.W. (1992) Hydrophobic surface protein masking by the opportunistic fungal pathogen *Candida albicans*. *Infection and Immunity*. **60:** 1499-1508.

Hazen K.C., Mandell G., Coleman E., and Wu G. (2000) Influence of fluconazole at subinhibitory concentrations on cell surface hydrophobicity and phagocytosis of *Candida albicans*. *FEMS Microbiology Letters*. **183:** 89-94.

Hazen K.C., Wu J.G., and Masuoka J. (2001) Comparison of the Hydrophobic properties of *Candida albicans* and *Candida dubliniensis*. *Infection and Immunity*. **69:** 779-786.

Henriques M., Azeredo J., and Oliveira R. (2004) Adhesion of *Candida albican*s and *Candida dubliniensis* to acrylic and hydroxipapatite. *Colloids and surfaces B: Biointerfaces*. **33:** 235-241.

Henriques M., Gasparetto K., Azeredo J., and Oliveira R. (2002) Experimental methodology to quantify *Candida albicans* cell surface hydrophobicity. *Biotechnology Letters*. **24:** 1111-1115.

Hjerten S, Rosengren J., and Pahlam S. (1974) Hydrophobic interaction chromatography. The synthesis and the use of some alkyl and aryl derivative of agarose. *Journal of Chromatography*. **101:** 281-288.

Hoffman M.P. and Haidaris C.G. (1993) Analysis of *Candida albicans* adhesion to salivary mucin. *Infection and Immunity*. **61:** 1940-1949.

Holmes A.D., Bandara B.M.K., and Cannon R.D. (2002) Saliva promotes *Candida albicans* adherence to human epithelial cells. *Journal of Dental Research*. **81:** 28-32.

Hsu B. and Huang C. (2002) Influence of ionic strengh and pH on hydrophobicity and zeta potential of *Giardia* and *Cryptosporidium*. *Colloids and surfaces A: physicochemical and engineering aspects*. **201:** 201-206.

Hube B., Sanglard D., Odds F.C., Hess D., Monod M., Schafer W., Brown A.J.P., and Gow N.A.R. (1997) Gene disruption of each of the secretory aspartyl proteinase genes SAP1, SAP2 and SAP3 in *Candida albicans* attenuates virulence. *Infection and Immunity*. **65:** 3529-3538.

Hunter P.R. and Fraser C. (1987) Use of modified resistrogram to type *Candida albicans* isolated from cases of vaginitis and from faeces in the same geographical area. *Journal of Clinical Pathology*. **40:** 1159-1161.

Jabra-Rizk M.A., Brenner T.M., Romagnoli M., Baqui A.A.M.A., Merz W.G., Falkler W.A., and Meiller T.F. (2001a) Evaluation of a reformulated CHROMagar Candida. *Journal of Clinical Microbiology*. **39:** 2015-2016.

Jabra-Rizk M.A., Falkler W.A., JR, Merz W.G., Kelley J.I., Baqui A.A.M.A., and Meiller T.F. (1999) Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. *Journal of Clinical Microbiology*. **37:** 1464-1468.

Jabra-Rizk M.A., Falkler W.A., Merz W.G., Baqui A.A.M.A., Kelley J.I., and Meiller T.F. (2001b) Cell surface hydrophobicity-associated adherence of *Candida*

dubliniensis to human buccal epithelial cells. *Revista Iberoamicana de Micologia*. **18:** 17-22.

Jabra-Rizk M.A., Falkler W.A., Merz W.G., and Meiller T.F. (2001c) New assay for measuring cell surface hydrophobicities of *Candida dubliniensis* and *Candida albicans*. *Clinical and Diagnostic Laboratory Immunology*. **8:** 585-587.

Jacobs P.H. and Nall L. (1997) Fungal disease. Biology, Immunology and diagnosis,Marcel Dekker, Inc. (Eds), New York

James A.M. (1991) Charge properties of microbial cell surfaces. *In*:Mozes N., Handley P.S., Busscher H.J., and Rouxhet P.G. (Eds).*Microbial cell surface analysis. Structural and physicochemical methods,* VCH Press, New York, pp 223-262.

Jarvis W.R. (1995) Epidemology of nosocomial fungal infections with emphasis on *Candida* species. *Clinical and Infectious Diseases*. **20:** 1526-1530.

Jass J., Surman S., and Walker J.T. (2003) Microbial biofilms in Medicine. *In*:Jass J., Surman S., and Walker J.T. (Eds).*Medical Biofilms,*1-28.

Jenkinson H.F. and Douglas L.J. (2002) Interactions between *Candida* species and bacteria in mixed infections. *In*:Brogden K.A. and Guthmiller J.M. (Eds).*Polymicrobial diseases,* ASM Press, 357-373.

Jin Y., Samaranayke L.P., Samaranayke Y.H., and Yip H.K. (2004) Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Archives of Oral Biology*. **49:** 789-798.

Jin Y., Yip H.K., Samaranayke Y.H., Yau J.Y., and Samaranayke L.P. (2003) Biofilm forming ability of *Candida albicans* is unlikely to contribute to high oral yeast carriage in human immunodeficiency virus-infection. *Journal of Clinical Microbiology*. **41:** 2961-2967.

Johnson L.E., Meyers N.L., and Jackson G.A. (2000) Inhibition of the growth of *Streptococcus* mutans to a glass surface. Proceedings of 147th Ordinary Meeting,SGM, pp 10.

Karlsson J.O.M. and Toner M. (2000) Cryopreservation. *In*:Lanza R.P., Langer R., and Vacanti J. (Eds).*Principles of Tissue Engineering,* Academic Press, 293-307.

Kennedy M.J. and Sandin R.L. (1988) Influence of growth conditions on *Candida albicans* adhesion, hydrophobicity and cell wall ultrastructure. *Journal of Medical and Veterinary Mycology*. **26:** 79-92.

Kimura L.H. and Pearsall N.N. (1978) Adherence of *Candida albicans* to human buccal epithelial cells. *Infection and Immunity*. **21:** 64-68.

Kuhn D.M, Balkis M., Chandra J., Mukherjee P.K., and Ghannoum M.A. (2003) Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. *Journal of Clinical Microbiology*. **41:** 506-508.

Kuhn D.M, Chandra J., Mukherjee P.K., and Ghannoum M.A. (2002a) Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrobial Agents and Chemotherapy*. **46:** 1773-1780.

Kuhn D.M, Chandra J., Mukherjee P.K., and Ghannoum M.A. (2002b) Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infection and Immunity*. **70:** 878-888.

Kumamoto C.A. (2002) Candida biofilms. *Current Opinion in Microbiology*. **5:** 608-611.

Lee K.H., Yoon M.S., and Chun W.H. (1997) The effects of monoclonal antibodies against iC3b receptors in mice with experimentally induced disseminated candidiasis. *Immunology*. **92:** 104-110.

Lewis K. (2001) Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*. **45:** 999-1007.

Li, X., Yan, Z., and Xu, J. (2003) Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. *Microbiology*. **149:** 353-362.

Liljemark W.F. and Gibbons R.J. (1973) Suppression of *Candida albicans* by human streptococci in gnotobiotic mice. *Infection and Immunity*. **8:** 846-

Lindhal M., Faris A., Wadstrom T., and Hjerten S (1981) A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. *Biochemistry and Biophysics*. **671:** 471-476.

Lischewski A., Kretschmar M., Hof H., Amann R., Hacker J., and Morschauser J. (1997) Detection and identification of *Candida* Species in experimentally infected tissue and human blood by rRNA-specific Fluorescent In Situ Hibridization. *Journal of Clinical Microbiology*. **35:** 2943-2948.

Lopes M.A., Knowles J.C., Santos J.D., Monteiro F.J., and Olsen I. (2000) Direct and indirect effects of P2O5 glass reinforced-hydroxyapatite composites on the growth and function of osteoblas-like cells. *Biomaterials*. **21:** 1165-1172.

Marsh P.D. (2000) Oral ecology and its impact on oral microbial diversity. *In*:Kuramitsu H.K. and Ellen R.P. (Eds).*Oral Bacterial Ecology: The molecular basis,* Horizon Scientific Press, Wymondham, pp 11-65.

Marshall K.C. (1991) The importance of studying microbial cell surfaces. *In*:Mozes N., Handley P.S., Busscher H.J., and Rouxhet P.G. (Eds).*Microbial cell surface analysis. Surface and physicochemical methods,* VCH Publishers, New York, pp 5-19.

Martin M.V. (1989) A comparison of fluconazole and ketaconazole in the treatment of rat palatal candidosis. *Journal of Medical and Veterinary Mycology*. **27:** 63-70.

Masuoka J. and Hazen K.C. (1997) Cell wall protein mannosylation determines *Candida albicans* cell surface hydrophobicity. *Microbiology*. **143:** 3015-3021.

Masuoka J., Wu G., Glee P.M., and Hazen K.C. (1999) Inhibition of *Candida albicans* attchment to extracellular matrix by antibodies which recognize hydrophobic cell wall proteins. *FEMS Immunology and Medical Microbiology*. **24:** 421-429.

McCullough M.J., Ross B.C., and Reade P.C. (1995) Characterization of genetically distinct subgroup of *Candida albicans* strains isolated from oral cavities of patients infected with Human Immunodeficiency Virus. *Journal of Clinical Microbiology*. **33:** 696-700.

McCullough M.J., Ross B.C., and Reade P.C. (1996) *Candida albicans*: a review of its history, taxonomy, epidomiology, virulence attributes, and methods of strain differentiation. *International Journal of Oral and Maxillofacial Surgery*. **25:** 137-144.

Meletiadis J., Meis J.F.G., Mouton J.W., Donnelly J.P., and Verweij P.E. (2000) Comparison of NCCLS and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) methods of in vitro susceptibility testing of filamentous fungi and development of a new simplified method. *Journal of Clinical Microbiology*. **38:** 2949-2954.

Meletiadis J., Mouton J.W., Meis J.F.G., Bouman B.A., Donnelly J.P., Verweij P.E., and Network E. (2001a) Colorimetric assay for antifungal susceptibility testing of Aspergillus species. *Journal of Clinical Microbiology*. **39:** 3402-3408.

Meletiadis J., Mouton J.W., Meis J.F.G., Bouman B.A., Donnelly P.J., Verweij P.E., and Network E. (2001b) Comparison of spectrophotometric and visual readings of NCCLS method and evaluation of a colorimetric method based on reduction of soluble tetrazolium salt, 2,3-bis{2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide}, for antifungal susceptibility testing of *Aspegillus* species. *Journal of Clinical Microbiology*. **39:** 4256-4263.

Merritt K., Gaind A., and Anderson J.M. (1998) Detection of bacterial adherence on biomedical polymers. *Journal of Biomedical Materials Research*. **39:** 415-422.

Millsap K.W., Bos R., Busscher H.J., and van der Mei H.C. (1999a) Surface aggregation of *Candida albicans* on glass in the absence and presence of adhering *Streptococcus gordonii* in a parallel-plate flow chamber: a surface thermodynamical analysis based on acid-base interactions. *Journal of Colloid and Interface Science*. **212:** 495-502.

Millsap K.W., Bos R., van der Mei H.C., and Busscher H.J. (1999b) Adhesion and surface-aggregation of *Candida albicans* from saliva on acrylic surfaces with adhering bacteria as studied in a parallel plate flow chamber. *Antonie van Leeuwenhoek*. **75:** 351-359.

Millsap K.W., van der Mei H.C., Bos R., and Busscher H.J. (1998) Adhesive interactions between medicaly important yeasts and bacteria. *FEMS Microbiology Reviews*. **21:** 321-336.

Moran G.P., Sullivan D.J., and Coleman D.C. (2002) Emergence of non-*Candida albicans Candida* species as pathogens. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D. C., pp 37-53.

Mosmann T.R. (1983) Rapid colorimetric assay for cellular growth and survival. *Journal of Immunology Methods*. **65:** 55-63.

Naglik J.R., Newport G., White T.C., Fernandes-Naglik L.L., Greenspan J.S., Greenspan D., Sweet S.P., Chalacombe S.J., and Agabian N. (1999) In Vivo Analysis of secreted Aspartyl Proteinase expression in human oral Candidiasis. *Infection and Immunity*. **67:** 2482-2490.

Nair R.G. and Samaranayke L.P. (1996a) The effect of oral commensal bacteria on candidal adhesion to denture acrylic surfaces. *APMIS*. **104:** 339-349.

Nair R.G. and Samaranayke L.P. (1996b) The effect of oral commensal bacteria on candidal adhesion to human buccal epithelial cells in vitro. *Journal of Medical Microbiology*. **45:** 179-185.

National Committee for clinical laboratory standars (1997) National Comittee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard NCCLS document M27-A. *Wayne, Pa*.

Nikawa H., Chen J., Hamada T., Nishimura M., and Polyzois G. (2001a) *Candida albicans* colonization on thermal cycled maxillofacial polymeric materials in vitro. *Journal of Oral Rehabilitation*. **28:** 526-533.

Nikawa H., Egusa H., Makihira S., Nishiruma M., Ishida K., Furukawa M., and Hamada T. (2002) A novel technique to evaluate the ahdesion of *Candida* species to gingival epithelial cells. *Mycoses*. **46:** 384-389.

Nikawa H., Hamada T., Murata H., and Subiwahjudi A. (1998) The effect of saliva or serum on *Streptococcus mutans* and *Candida albicans* colonization of hydroxylapatite beads. *Journal of Dentistry*. **26:** 31-37.

Nikawa H., Jin L.J., Hamada T., Makihira S., and Polyzois G. (2001b) *Candida albicans* growth on thermal cycled materials for maxillofacial prostheses in vitro. *Journal of Oral Rehabilitation*. **28:** 755-765.

Nikawa H., Nishimura H., Hamada T., Kumagai H., and Samaranayke L.P. (1997) Effects of dietary sugars and, saliva and serum on *Candida* biofilm formation on acrylic surfaces. *Mycopathologia*. **139:** 87-91.

Nikawa H., Nishimura M., Makihira S., Hamada T., Sadamori S., and Samaranayke L.P. (2000) Effect of serum concentration of *Candida* biofilm formation on acrylic surfaces. *Mycoses*. **43:** 139-143.

O'Sullivan J.M., Jenkinson H.F., and Cannon R.D. (2000) Adhesion of *Candida albicans* to oral *streptococci* is promoted by selective adsorption of salivary proteins to the streptococcal. *Microbiology*. **146:** 41-48.

Odds F.C. (1987) *Candida* infections: an overview. *Critical Reviews in Microbiology*. **15:** 1-5.

Oliveira R. (1997) Understanding Adhesion: a means for preventing fouling. *Experimental Thermal and Fluid Science*. **14:** 316-322.

Palsson B. and Bhatia S. (2004) Tissue engineering,Pearson Prentice Hall (Eds),

Panagoda G.J., Ellepola A.N.B., and Samaranayke L.P. (2001) Adhesion of *Candida parapsilosis* to epithelial and acrylic surfaces correlates with cell surface hydrophobicity. *Mycoses*. **44:** 29-35.

Panagoda G.J., Ellepola A.N.B., and Samaranayke L.P. (1998) Adhesion to denture acrylic surfaces and relative cell-surface hydrophobicity of *Candida parapsilosis* and *Candida albicans*. *APMIS*. **106:** 736-742.

Papaioannou W. (1998) The adhesion of *Porphyromonas gingivalis* and periodontitis. phDLeuven University

Pendrak M.L. and Klotz S.A. (1995) Adherence of *Candida albicans* to host cells. *FEMS Microbiology Letters*. **129:** 103-114.

Penha S.S., Birman E.G., Silveira F.R.X., and Paula C.R. (2000) Frequency and enzymatic activity (proteinase and phospolipase) of *Candida albican*s from edentolous patients, with and without denture stomatitis. *Pesquisa Odontontologica do Brasil*. **14:** 119-122.

Pereiro M., Losada A., and Toribio J. (1997) Adherence of *Candida albicans* strains isolated from AIDS patients. Comparison with pathogenic yeasts isolated from patients without HIV infection. *British Journal of Dermatology*. **137:** 76-80.

Persi M.A., Burnham J.C., and Duhring J.L. (1985) Effects of carbon dioxide and pH on adhesion of *Candida albicans* to vaginal epithelial cells. *Infection and Immunity*. **50:** 82-90.

Pfaller M.A. (1996) Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. *Clinical and Infectious Diseases*. **22:** 89-94.

Pfaller M.A., Houston A., and Coffmann S. (1996) Application of CHROMagar Candida for Rapid Screening of clinical specimens for *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida* (*Trulopsis*) *glabarata*. *Journal of Clinical Microbiology*. **34:** 58-61.

Pfaller M.A., Messer S.A., and Hollis R.J. (1995) Variations in DNA subtype, antifungal susceptibility and slime production among clinical isolates of *Candida parapsilosis*. *Diagnosis in Microbiology and Infectious Diseases*. **21:** 9-14.

Pfaller M.A. and Wenzel R. (1992) Impact of the changing epidemiology of fungal infections in the 1990s. *European Journal of Clinical and Microbiology Infectious Diseases*. **11:** 287-291.

Phan Q.T., Belanger P.H., and Filler s.G. (2000) Role of hyphal formation in interactions of *Candida albicans* with Endothelial Cells. *Infection and Immunity*. **68:** 3485-3490.

Pitts, B., Hamilton, M. A., Zelver, N., and Stewart, P. S. (2003) A microtiter-plate screening method for biofilm disinfection and removal. *Journal of Microbiological Methods*. **54:** 269-276.

Pizzo G., Giuliana G., Milici M.E., and D'Angelo M. (2001) Effect of antimicrobial mouthrinses on the in vitro adhesion of *Candida albicans* to human buccal epithelial cells. *Clinical and Oral Investigation*. **5:** 172-176.

Pla J., Gil C., Monteoliva L., Navarro-García F., Sanchez M., and Nombela C. (1996) Understandig *Candida albicans* at the Molecular Level. *Yeast*. **12:** 1677-1702.

Potera C. (1999) Forging a link between biofilms and disease. *Science*. **283:** 1837-1838.

Prasad R. (1991) *Candida albicans*, Springer-Verlag (Eds), Berlin

Raad, I., Chatzinikolaou, I., Chaiban, G., Hanna, H., Hachem, R., Dvorak, T., Cook, G., and Costerton, W. (2003) In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. *Antimicrobial Agents and Chemotherapy*. **47:** 3580-3585.

Radford D.R., Sweet S.P., Challacombe S.J., and Walter J.D. (1998) Adherence of *Candida albicans* to denture-base materials with different surface finisshes. *Journal of Dentistry*. **26:** 577-583.

Ramage G., Walle K.V., Bachmann S.P., Wickes B.L., and López-Ribot J.L. (2002) In vitro pharmodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrobial Agents and Chemotherapy*. **46:** 3634-3636.

Ramage G., Walle K.V., and López-Ribot J.L. (2001) Biofilm formation by *Candida dubliniensis*. *Journal of Clinical Microbiology*. **39:** 3234-3240.

Rex J.H., Pfaller M.A., Walsh T.J., Chaturvedi V., Espinel-ingroff A., Ghannoum M.A., Gosey L.L., Odds F.C., Rinaldi M.G., Sheehan D.J., and Warnock D.W. (2001) Antifungal susceptibility testing: practical aspects and current challenges. *Cllinical Microbiology Reviews*. **14:** 643-658.

Riesselman M.H., Hazen K.C., and Cuttler J.E. (2000) Determination of antifungal MICs by a rapid susceptibility assay. *Journal of Clinical Microbiology*. **38:** 333-340.

Robin C.P. (1853) Histoire Naturelle des vegeteaus. Parasites qui croissent sur l'homme et sur les animaux vivants. *Balliere, Paris, France*.

Robinson L.B., Wichelhausen R.B., and Roizman B. (1956) Contamination of human cell cultures by pleuro-pneumonia-like organisms. *Science*. **124:** 1147-1148.

Rodrigues A.G., Mardh P.A., Pina-Vaz C., Martinez-de-Oliveira J., and Fonseca A.F. (1999) Germ tube formation changes surface hydrophobicity of *Candida* cells. *Infectious Diseases in Obstetrics and Gynecology*. **7:** 222-226.

Roehm N.W., Rodgers G.H., Hatfield S.M., and Glasebrook A.L. (1991) An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *Journal of Immunology Methods*. **142:** 257-265.

Rouxhet P.G. and Genet M.J. (1991) Chemical composition of the microbial cell surface by X-ray photoelectron spectroscopy. *In*:Nava Mozes, Pauline S. Handley Henk J. Busscher Paul G. Rouxhet (Eds).*Microbial cell surface analysis. Structural and physicochemical methods,* VCH, New York, pp 204-217.

Rouxhet P.G., Mozes N., Dengis P.B., Dufrêne Y.F., Gerin P.A., and Genet M.J. (1994) Application of X-ray photoelectron spectroscopy to microorganisms. *Colloids and surfaces B: Biointerfaces*. **2:** 347-369.

Samaranayke L.P. and MacFarlane T.W (1980) An in-vitro study of the adherence of *Candida albicans* to acrylic surfaces. *Archives of Oral Biology*. **25:** 603-609.

Samaranayke L.P. and MacFarlane T.W (1982) The effect of dietary carbohydrates on the in-vitro adhesion of *Candida albicans* to epithelial cells. *Journal of Medical Microbiology*. **15:** 511-517.

Samaranayke L.P., McCourtie J., and MacFarlane T.W (1980) Factors affecting the in-vitro adherence of *Candida albicans* to acrylic surfaces. *Archives of Oral Biology*. **25:** 611-615.

Samaranayke Y.H., Wu P.C., Samaranayke L.P., and So M. (1995) Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelial and denture acrylic surfaces. *APMIS*. **103:** 707-713.

San Millan R., Elguezabal N., Regulez P., Moragues M.D., Quindos G., and Ponton J. (2000) Effect of salivary secretory IgA on the adhesion of *Candida albicans* to polysterene. *Microbiology*. **146:** 2105-2112.

Sanglard D. and Bille J. (2002) Current understanding of the modes of action of and resistance mechanisms to conventional and emerging antifungal agents for treatment of *Candida* infections. *In*:Calderone R.A. (Eds).*Candida and Candidiasis,* ASM Press, Washington D.C., pp 349-383.

Schoofs A., Odds F.C., Colebunders R., Ieven M., and Goosens H. (1997) Use of specialised isolation media for recognition and identification of *Candida dubliniensis* isolates from HIV-infected patients. *European Journal of Clinical and Microbiology Infectious Diseases*. **16:** 296-300.

Segal E. and Sandovsky-Losica H. (1995) Adhesion and interaction of *Candida albicans* with Mammalian Tissues in Vitro and in Vivo. *Methods in Enzymology*. **253:** 439-452.

Sen B.H., Safavi K.E., and Spangberg L.S.W. (1997) Colonization of *Candida albicans* on cleaned human dental hard tissues. *Archives of Oral Biology*. **42:** 513-520.

Skerl K.G., Calderone R.A., Segal E., Sreevalsan T., and Scheld W.M. (1984) In vitro binding of *Candida albicans* yeast cells to human fibronectin. *Canadian Journal of Microbiology*. **30:** 221-227.

Staib F. (1969) Proteolysis and pathogenicity of *Candida albicans* strains. *Mycopathology and Mycology Application*. **37:** 345-348.

Staib P., Kretschmar M., and Nichterlein T. (1999) Host-induced stage.specific virulence gene activation in *Candida albicans* during infection. *Molecular Microbiology*. **32:** 533-546.

Stepanovic S., Vukovic D., Dakic I., Savic B., and Svabic-Vlahovic M. (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*. **40:** 175-179.

Sturtevant J. and Calderone R. (1997) *Candida albicans* adhesins: Biochemical aspects and virulence. *Revista Iberoamicana de Micologia*. **14:** 90-97.

Sullivan D.J. and Coleman D.C. (1998) *Candida dubliniensis*: Characteristics and Identification. *Journal of Clinical Microbiology*. **36:** 329-334.

Sullivan D.J., Haynes K., Bille J., Boerlin P., Rodero L., Lloyd S., Henman M., and Coleman D.C. (1997) Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. *Journal of Clinical Microbiology*. **35:** 960-964.

Sullivan D.J., Westerneng T.J., Haynes K.A., Bennett D.E., and Coleman D.C. (1995) *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology*. **141:** 1507-1521.

Summers D.F., Gollman A.P., and Hasenclever H.F. (2002) Polysaccharide antigens of the *Candida* cell wall. *J.Immunol.* **92:** 491-499.

Suzuki S. (2002) Serological differences among the pathogenic *Candida* spp. *In*:Calderone R.A. (Eds). *Candida and Candidiasis,* ASM Press, Washington D. C., pp 29-36.

Taylor R., Verran J., and Maryan C.J. (1998) Retention of oral microorganisms on cobalt-chromium alloy and dental acrylic resin with different surface finishes. *Journal of phrostetic dentistry*. **5:** 592-597.

Tronchin G., Robert R., and Bouali A. (1987) Immunocytochemical localization of in vitro binding of human fibrinogen to *Candida albicans* germ tube and mycelium. *Annuel Instute Pasteur / Microbiology*. **138:** 177-187.

Ueta E., Tanida T., Doi S., and Osaki T. (2000) Regulation of *Candida albicans* growth and adhesion by saliva. *The Journal Laboratory and Clinical Medicine*. **136:** 67-73.

Umazume M., Ueta E., and Osaki T. (1995) Reduced inhibition of *Candida albicans* adhesion by saliva from patients receiving oral cancer therapy. *Journal of Clinical Microbiology*. **33:** 432-439.

van der Mei H.C., Bos R., and Busscher H.J. (1998) A reference guide to microbial cell surface hydrophobicity based on cantact angles. *Colloids and surfaces B: Biointerfaces*. **11:** 213-221.

van der Mei H.C., Vries J., and Busscher H.J. (2000) X-ray photoelectron spectroscopy for the study of microbial cell surfaces. *Surface Science Reports*. **39:** 1-24.

van Oss C.J., Chaudhury M.K., and Good R.J. (1987) Monopolar surfaces. *Advances in Colloid in Interface Science*. **28:** 35-64.

van Oss C.J. and Giese R.F. (1995) The hydrophilicity and hydrophobicity of clay minerals. *Clay Minerals*. **2:** 347-369.

Verran J., Lees G., and Shakespeare A.P. (1991) The effect of surface roughness on the adhesion of *Candida albicans* to acrylic. *Biofouling*. **3:** 183-192.

Verran J. and Maryan C.J. (1997) Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. *Journal of phrostetic dentistry*. **77:** 535-539.

Verran J. and Motteram K.L. (1987) The effect of adherent oral streptococci on the subsequent adherence of *Candida albicans* to acrylic in vitro. *Journal of Dentistry*. **15:** 73-76.

Vidotto V., Mantoan B., Pugliese A., Ponton J., Quindos G., Aoki S., and Ito-Kuwa S. (2003) Adherence of *Candida albicans* and *Candida dubliniensis* to buccal and vaginal cells. *Revista Iberoamicana de Micologia*. **20:** 52-54.

Warnock D.W. and Johnson E.M. (1997) Azole drug resistance as a cause of treatment failure in patients with Candidosis. *In*:Jacobs P.H. and Nall L. (Eds).*Fungal disease. Biology, Immunology, and diagnosis,* Dekker, New York, pp 487-499.

Waters M.G.J., Williams D.W., Jagger R.G., and Lewis M.A.O. (1997) Adherence of *Candida albicans* to experimental denture soft lining materials. *The Journal of Prosthetic Dentistry*. **77:** 306-312.

Watts H.J., Cheah F.S.H., Hube B., Sanglard D., and Gow N.A.R. (1998) Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. *FEMS Microbiology Letters*. **159:** 129-135.

Weerkamp A.H. and McBride B.C. (1980) Adherence of salivarius HB and HB-7 to oral surfaces and saliva coated hydroxyapatite. *Infection and Immunity*. **30:** 150-158.

Wenzel R. (1995) Nosocomial candiddemia: risk fectors and attributable mortality. *Clinical and Infectious Diseases*. **20:** 1531-1534.

Whelan W.L. and Magee P.T. (1981) Natural heterozygosity in *Candida albicans*. *The Journal of Bacteriology*. **145:** 896-903.

Williams D.W., Waters M.G.J., Potts A.J.C., and Lewis M.A.O. (1998) A novel technique for assessment of adherence of *Candida albicans* to solid surfaces. *Journal of Clinical Pathology*. **51:** 390-391.

Wimpenny J.W.T. and Colasanti R. (1997) A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *FEMS Microbiology Ecology*. **22:** 1-16.

Zepelin M.B., Niederhaus T., Gross U., Seibold M., Monod M., and Tintelnot K. (2002) Adherence of different *Candida dubliniensis* isolates in the presence of fuconazol. *AIDS*. **16:** 1237-1244.